ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PRODUCTION AND OPTIMIZATION OF CAROTENOIDS AND LIPIDS FROM OLEAGINOUS RED YEAST *RHODOSPORIDIUM TORULOIDES Y27012*

M.Sc. THESIS

Furat Alakraa

Department of Food Engineering

Food engineering programme

JUNE 2014

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

PRODUCTION AND OPTIMIZATION OFCAROTENOIDS AND LIPIDS FROM OLEAGINOUS RED YEAST *RHODOSPORIDIUM TORULOIDES Y27012*

M.Sc. THESIS

Furat Alakraa
(506111526)

Department of Food Engineering

Food Engineering Programme

Thesis Advisor: Ass. Prof. Dr. Neşe ŞAHİN YEŞİLÇUBUK

JUNE 2014

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

RHODOSPORIDIUM TORULOIDES Y 27012 MAYASINDAN KAROTENOİD VE TEK HÜCRE YAĞI ÜRETİMİ VE OPTİMİZASYONU

YÜKSEK LİSANS TEZİ

Furat Alakraa
(506111526)

Gıda Mühendisliği Bölümü

Gıda Mühendisliği Programı

Tez Danışmanı: Yrd. Doç. Dr. Neşe ŞAHİN YEŞİLÇUBUK

HAZİRAN 2014

Furat ALAKRAA, a **M.Sc.** student of ITU **Graduate School of Science Engineering and Technology** student ID 506111526, successfully defended the **thesis** entitled "**LIPID VE CAROTENOIDS PRODUCTION AND OPTIMIZATION FROM OLEAGINOUS RED YEAST *RHODOSPORIDIUM TORULOIDES Y27012***", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Yrd. Doç. Dr. NEŞE Şahin YEŞİLÇUBUK** ….....

İstanbul Technical University

Jury Members : **Yrd. Dr.Dilara Erdil** ….....
İstanbul Technical University

Prof. Dr.Hikmet Boyacıoğlu ….....
Okan University

Date of Submission : 5 May 2014
Date of Defense : 3 June 2014

FOREWORD

My sincere and deep gratitude is given to my thesis advisor, Yrd. Doç. Dr.NEŞE ŞAHİN YEŞİLÇUBUK who did not only guided me with valuable advices throughout the course of my reserch, but also educates me to possess scientific mind thinking and proplem solving.

Grateful acknowledgment are to Ms. AYŞE SAYGUN for helpful advices, continuous support and suggestions on various aspects on my reserch work.

My acknowledgment and thanks are also extended to all of the people in Department of food engineering.

I would to acknowledgment my beatiful family for thier appreciation and everlastin love through my life

I would also like to thank my soulmate Ahmad Algohary for his encourgement, and help with his unconditional love.

Last but not least, I am grateful to my friends in every where for their love and invaluable encouragement, especially in my difficult days

May 2014

Furat Alakraa
Food Engineer

TABLE OF CONTENTS

Page

ABBREVIATIONS

ANOVA : Analysis of variance
C/N : Carbon to nitrogen ratio
CCD : Central composite design
DMSO : Dimethyl sulfoxide
FAME : Fatty acid methyl ester
GC : Gaz chromortography
HPLC : High performance liquid chromatography
P_m : Carotenoids concentration
RSM : Respond surface methodology
SCO : Single cell oil
SD : Standart deviation
TLC : Thin-Layer Chromatography
US : Ultrasonic method
X_m : Dried cell biomass
$Y_{p/x}$: Product yield

LIST OF TABLES

Page

LIST OF FIGURES

Page

PRODUCTION AND OPTIMIZATION OF CAROTENOIDS AND LIPIDS FROM OLEAGINOUS RED YEAST *RHODOSPORIDIUM TORULOIDES Y27012*

SUMMARY

The ability of certain microorganisms to accumulate high amount of lipid and carotenoids has been known for years. In the last years, real efforts have been made to study their pathways, and optimization of production conditions and culture medium. Microbial lipid compounds, known as single cell oils (SCO) have industrial interest due to their particular and precise properties.

Oily yeasts (OY) have been described to be able to accumulate lipid up to 20% of their cellular dry weight. These yeasts represent minor proportion of the total yeasts, but about 25% of them are able to produce 25% of lipid.

In recent years, increasing evidences for toxicological effects of synthetic colors have prompted regulatory agencies worldwide to drastically prune the list of permitted synthetic food colors. As a result of stringent rules and regulations applied to chemically synthesize purified pigments, and consumer preferences and growth of food industry, demand of carotenoids as safe and suitable coloring agents is on rise. Microorganisms offer economical production of carotenoids through biotechnological methods and provide an alternative to chemical synthesis. More than 600 different carotenoids are produced by plants, algae, bacteria, and fungi. However, only a few can be obtained in useful quantities by chemical synthesis, and extracted from their natural sources or obtained by microbial fermentation.

In this work, effect of medium additives on the production of carotenoids and lipids from R*hodosporidium toruloides* Y27012 were investigated and furthermore medium components were optimized by response surface methodology (RSM). In the first part of the study, two methods such as ultrasonic (US) assisted and HCl assisted extractrion techniques were compared with control technique (grinding) on carotenoids extraction yield from *Rhodosporidium toruloides* Y27012.HCl-assisted extraction of carotenoids was found to be the most effective method with a carotenoid yield of 977±43.25 µg/g and concentration of 31.24±0.78 mg/L, compared with US method and grinding (control).

In the second part of the study, various carbon sources, nitrogen sources and different carbon to nitrogen ratios (C/N) and additives were investigated in order to obtain the highest carotenoids production from *Rhodosporidium toruloides*Y27012. Lipid contents were also determined during the study.

Among the nitrogen sources that were examined, yeast extract was found to be the best nitrogen source for carotenoids yield and concentration (977.12 ± 43.25 µg/g and 31.24±0.78mg/L) followed by peptone and ammonium sulfate.

Also yeast extract gave the highest lipid content and lipid yield (41.71±1.02 %,15.23±2.06g/L).

Assimilation of different sugars as carbon source by the red yeast *Rhodosporidium toruloidesY27012*for its growth and pigmentation showed that highest carotenoids yield was 977.12±43.28 µg/g when glucose was used as carbon source at C/N ratio of 40, whereas It was noticed from this research that no clear difference between lipid content was found when glucose and glycerol sources were used. Glucose gave the highest lipid concentration 15.23±2.06 g/L compared with glycerol (6.16±0.40 g/L) which gave the lowest one compared with glucose and xylose.

The effect of C/N ratio was examined to study its effect on lipid and carotenoids production. The maximum carotenoids production was obtained at C/N ratio of 20 (24.76±1.78 g/L, 1001±17.87 µg/g) compared with low medium nitrogen contents at C/N ratio of 40 and 60. But at this ratio lipid contents and lipid yields were found to be low. It was noticed that lipid content increased with the increase in C/N (low nitrogen), and the highest lipid content was noticed at high C/N ratio 60 (49.83±2.53%, 16.98±0.79 g/L).

Effect of additives on carotenoids and lipid production at C/N ratio of 20 and ethanol addition (10 g/L) increased the carotenoid yield with no change in carotenoid concentration compared with control sample (1732.17±39.45µg/g, 22.92±0.95 mg/L), lipid content increased until 44.92±5.43% from 40.28±2.44% in control sample. But the lipid concentration was lower (8.59±1.05 g/L) compared with the control one.

It was noticed that addition of acetic acid (5 g/L) made a huge increase in lipid content but the carotenoids were not detected when acetic acid was added.

When some additives were added at low concentration such as 0.1% (v/v), they did not have any effect on carotenoid yield, but carotenoid concentration was still almost the same around 20 mg/L. When Tween 80 and Tween 20 were added at high concentration such as 1% (v/v) and glucose was adjusted to have C/N ratio of 20, an increase in carotenoid yield and carotenoid concentration were observed. Tween 80 gave 25±2.07 mg/L, 1014.46±68.44 µg/g carotenoids yield and concentration, respectively. Although Tween 20 gave 25.26±1.19 mg/L which was higher than control sample without any activators, but the carotenoid yields was almost the same with littile difference (1002.79±46.87 µg/g) compared with the control sample (1001.51±17.87 µg/g) with decrease in cell biomass. So at low glucose concentrations to have C/N ratio of 20, Tween20, Tween 80 at 1% (v/v) increased the carotenoid concentration with little increase in carotenoid yield compared with control sample.

Adding Tween 80 and Tween 20 at 0.1% and 1% (v/v) caused increase in lipid content, and the highest lipid content reached to 66.33±1.38 % when Tween 80 was added at 1% (v/v). Whereas when cotton seed oil and linseed oil were used at 0.1% and 1% (v/v), *Rhodosporidium toruloides* Y27012 didn't produce any carotenoids and the medium was still without any color even after 5 days of fermentatio

In the third part of the study, response surface methodology (RSM) was applied for optimizing the medium additives for the production of biomass, carotenoids and lipids. RSM can not only improve growth and production, but also reduce process variability, development time, and overall costs.

The graphic plot of predicted values by the model vs. observed experimental values showed a linear distribution for biomass , lipid content, and carotenoid yield(R^2=0.947, 0.959, and 0.921, respectively) for the response, This indicated that up to 92 ~ 96 % of the variations in biomass, lipids , and carotenoids can be explained by these equations.

The model of biomass was highly appropriate for the prediction since the Fmodel values was high compared to the F table.

It is obsorved that the most important effects on biomass incorporation was found as ethanol. "Glucose" , "ethanol*ethanol" , and "glucose* ethanol" do not have any effect on biomass as ($p>0.05$).

Wherease "glucose*glucose", "yeast extract*yeast extract", "ethanol* ethanol", and "yeast extract*ethanol" have an effect on lipid content as ($p<0.05$).

While "yeast extract*yeast extract", "ethanol* ethanol", and "glucose*yeast extract" have effects on carotenoid yield .

It was noticed after optimization by RSM that the maximum biomass (18.78 g/L) was obtained at glucose of 9.2819 g/L and decreased yeast extract at 1.78 g/L , alsomaximum lipid content (75.1405%) at decreased yeast extract ratio (1.77g/L).The maximum carotenoids yield (1680.25 µg/g) was obtained at glucose concentration of 6.43 g/L and relatively high yeast extract concentration of (3.77 g/L)

After optimization by RSM, individual carotenoids and fatty acid compositions were determined by high performance liquid chromatography (HPLC) and gas cgromatography (GC).

The fatty acid composition showed that four major constituent fatty acids were noticed oleic acid (18:1), palmitic acid (16:0), stearic acid (18:1) and linoleic acid (18:2). Oliec acid was noticed at 38.54% followed by palmitic acid (20.82%) then palmitoleic acid (12.71%), linoleic acid (12.01%), steatric acid(4.4%) palmitoleic acid. The rest fatty acid were at low ratios.

RHODOSPORIDIUM TORULOIDESY27012 MAYASINDAN KAROTENOİD VE TEK HÜCRE YAĞI ÜRETİMİ VE OPTİMİZASYONU

ÖZET

Bazı mikroorganizmaların yüksek miktarda lipit ve karotenoidleri depolama kabiliyeti yıllardır bilinmektedir. Son yıllarda yollarının incelenmesi ve üretim koşulları ve kültür ortamlarının optimizasyonu için gerçek çabalar gösterildi. Tek hücreli yağlar (SCO) olarak bilinen mikrobiyal lipit bileşikleri özel ve hassas nitelikleri nedeniyle endüstriyel ilgiye sahiptirler.

Yağlı mayalar (OY) hücresel kuru ağırlıklarının %20'sine kadar lipit depolama kabiliyetine sahip olarak tanımlanmıştır. Bu mayalar toplam mayaların küçük bir bölümünü teşkil eder fakat yaklaşık %25'leri lipidin %25'ini üretebilirler.

Son yıllarda, sentetik renklerin toksikolojik etkileri hakkında artan kanıtlar dünya çapında düzenleyici kuruluşların izin verilen gıda renkleri listesini radikal bir şekilde budamasına yol açmıştır. Kimyasal olarak sentezlenen saflaştırılmış pigmentlere uygulanan katı kurallar sonucunda ve tüketici tercihleri ile gıda endüstrisinin büyümesinin bir sonucu, güvenli ve uygun renklendiriciler olarak karotenoidlere olan talep artmaktadır. Mikroorganizmalar karotenoidlerin biyoteknolojik yöntemlerle ucuz üretimini sağlar ve kimyasal senteze bir alternatif oluşturur. 600'den fazla farklı karotenoid bitkiler, bakteri ve mantarlar tarafından üretilir. Ancak, sadece birkaçı kimyasal sentezle faydalı miktarlarda elde edilebilir ve doğal kaynaklarından çıkartılır veya mikrobiyal fermantasyonla elde edilebilir.
Bu çalışmada, karotenoidlerin ve *Rhodosporidium toruloides* Y27012'den lipitlerin üretimi üzerinde besiyeri katkılarının etkisi araştırılmış ve ayrıca besiyeri bileşenleri, yanıt yüzeyi metodolojisi (RSM) ile optimize edilmiştir.
Çalışmanın birinci bölümünde, ultrasonik (US) destekli ve HCI destekli extrasyon teknikleri olmak üzere karotenoidlerin *Rhodosporidium toruloides* Y27012. HCl-'den çıkarım verimi üzerinde 2 yöntem, kontrol tekniği (öğütme) ile mukayese edildi. Karotenoidlerin HCl destekli extrasyonu, 977±43.25 µg/g karotenoid verimi ve 31.24±0.78 mg/L konsantrasyonu ile US yöntemi ve öğütme (kontrol) ile mukayese edildiğinde en etkin yöntem olarak bulundu.

Çalışmanın ikinci bölümünde, muhtelif karbon kaynakları, azot kaynakları ve farklı karbon azot oranları (C/N) ve katkı maddeleri *Rhodosporidium toruloides* Y27012'den en yüksek karotenoid üretimini elde etmek için araştırıldı. Çalışma süresince lipit içerikleri de belirlendi.
İncelenen azot kaynakları arasında maya özütü karotenoid verimi ve konsantrasyonu (977.12 ± 43.25 µg/g ve 31.24±0.78mg/L) için en iyi azot kaynağı olarak tespit edildi, bunu pepton ve amonyum sülfat izledi. Maya özütü aynı zamanda en yüksek lipit içeriğini ve lipit verimini de verdi (41.71±%1.02, 15.23±2.06g/L).

Pigmantasyonu ve lipit üretimi için, farklı şekerlerin karbon kaynağı olarak kırmızı maya *Rhodosporidium toruloides* Y27012 tarafından asimilasyonunda en yüksek karotenoid veriminin, karbon kaynağı olarak glikozun C/N = 40 oranında kullanıldığı zaman, 977.12±43.28 µg/g olduğunu gösterdi; ancak bu araştırmadan glikoz ve gliserol kaynakları kullanıldığında lipit içerikte belirgin bir fark bulunmadığının farkına varıldı. Glikozun, gliserol ve ksiloz ile karşılaştırıldığında en düşük konsantrasyonu veren gliserol (6.16±0.40 g/L) ile mukayese edildiğinde en yüksek lipit konsantrasyonunu 15.23±2.06 g/L verdi

C/N oranının lipit ve karotenoidlerin üretimine etkisi üzerinde çalışılmak üzere incelendi. Maksimum karotenoid üretimi, C/N= 40 ve 60 oranındaki düşük ortam azot içeriğine kıyasla,C/N 20 oranında elde edildi (24.76±1.78 g/L, 1001±17.87 µg/g). Fakat bu oranda lipit içerikler ve lipit verimleri düşük bulundu. Lipit içeriğinin, C/N'deki artışla (düşük azot) arttığı fark edildi ve en yüksek lipit içerik C/N=60 yüksek oranında gözlemlendi (49.83±%2.53, 16.98±0.79 g/L).

Katkı maddelerinin C/N= 20 oranında ve etanol ilavesi (10 g/L) ile karotenoidler ve lipit üretimi üzerinde etkisi, kontrol numunesi ile karşılaştırıldığında (1732.17±39.45µg/g, 22.92±0.95 mg/L),karotenoid konsantrasyonunda değişiklik olmaksızın karotenoid verimini artırdı, kontrol numunesinde lipit içerik 40.28±%2.44'den 44.92±%5.43'e kadar arttı. Ancak lipit konsantrasyonu kontrol numunesininkine oranla daha düşüktü (8.59±1.05 g/L).

Asetik asit ilavesinin (5 g/L) lipit içerikte muazzam bir artış yarattığı fakat asetik asit ilave edildiğinde karotenoidlerin saptanmadığı not edildi.

Bazı katkı maddeleri %0.1 (v/v) gibi düşük konsantrasyonlarda ilave edildiğinde, karotenoid verimi üzerinde herhangi bir etkileri olmadı ve karotenoid konsantrasyonu hala hemen hemen yaklaşık 20 mg/L civarındaydı. % 1 (v/v) gibi yüksek konsantrasyonda Tween 80 ve Tween 20 ilave edildiğinde ve C/N=20 oranında olacak şekilde ayarlandığında, karotenoid veriminde ve karotenoid konsantrasyonunda bir artış gözlemlendi. Tween 80 sırasıyla 25±2.07 mg/L, 1014.46±68.44 µg/g karotenoid verimi ve konsantrasyonu verdi. Her ne kadar Tween 20, herhangi bir aktifleştiricisiz kontrol numunesinden daha yüksek olan 25.26±1.19 mg/L vermiş olsa da, karotenoid verimleri (1002.79±46.87 µg/g), kontrol numunesi ile mukayese edildiğinde küçük fark ve hücre biokütlesinde azalma ile hemen hemen aynı idi (1001.51±17.87 µg/g). Yani C/N=20 oranına sahip düşük glükoz konsantrasyonlarında, %1'lik (v/v) Tween20, Tween 80 kontrol numunesine kıyasla karotenoid veriminde az bir artışla beraber karotenoid konsantrasyonunu arttırdı.

%0.1 ve %1 (v/v)'de Tween 80 ve Tween 20 ilave edilmesi lipit içerikte artışa neden oldu ve %1 (v/v)'de Tween 80 ilave edildiğinde en yüksek lipit içeriği 66.33±%1.38'e ulaştı. Ancak, Pamuk tohumu yağı ve keten yağı %0.1 ve %1 (v/v)'de kullanıldıklarında, *Rhodosporidium toruloides* Y27012 herhangi bir karotenoid üretmedi ve ortamın fermantasyondan 5 gün sonra bile hala herhangi bir rengi yoktu.

Çalışmanın üçüncü bölümünde yanıt yüzeyi metodolojisi (RSM) biokütle, karotenoidler ve lipitlerin üretimi için ortam katkı maddelerini optimize etmek üzere uygulandı. RSM sadece büyüme ve üretimi geliştirmekle kalmaz aynı zamanda süreç değişkenliğini, gelişim süresini ve genel maliyetleri de düşürür.

Gözlemlenen deneysel değerlere karşın model tarafından öngörülen değerlerin grafik çizimi yanıt için biokütle, lipit içerik ve karotenoid verimi için tepki olarak lineer bir dağılım gösterdi (sırasıyla R^2=0.947, 0.959 ve 0.921).Bu, biokütle, lipitler ve karotenoidlerdeki %92 ~ 96'ya kadar olan değişimlerin bu denklemlerle açıklanabileceğini gösterdi.

F model değerleri F tablosuna kıyasla yüksek olduğundan, biokütlenin modeli öngörü için fazlasıyla uygundu.
Biokütle kaynaşması üzerinde en önemli etkilerin etanol olarak bulunduğu gözlemlendi. "Glikoz", "etanol*ethanol" ve "glikoz* etanol" (p> 0.05) olarak biyokütle üzerinde herhangi bir etkisi yoktur.
Oysaki "glikoz*glikoz", "maya özütü*maya özütü", "etanol* etanol", ve "maya özütü*etanol"'ün, (p> 0.05) olarak lipit içerik üzerinde bir etkisi vardır.
Diğer yandan "maya özütü*maya özütü", "etanol* etanol", ne "glikoz*maya özütü"'nün karotenoid verimi üzerinde etkileri vardır.
RSM ile optimizasyondan sonra azami biokütlenin (18.78 g/L), 9.2819 g/L'lik glikozda ve 1.78 g/L'e azaltılmış maya özütünde, aynı zamanda, azami lipit içeriğin (%75.1405) azaltılmış maya özütü oranında (1.77g/L) elde edildiği ortaya koyuldu. Azami karotenoid verimi (1680.25 µg/g) 6,43 g/L'lik glikoz konsantrasyonunda ve göreceli olarak yüksek maya özütü konsantrasyonunda elde edildi.
RSM ile optimizasyondan sonra münferit karotenoidler ve yağ asidi bileşimleri yüksek performanslı sıvı kromatografisi (HPLC) ve gaz kromatografisi (GC) ile saptandı.
Yağ asidi bileşimi dört ana yağ asidi bileşeni ortaya koyulduğunu gösterdi; oleik asit (18:1), palmitik asit (16:0), stearik asit (18:1) ve linoleik asit (18:2). Oleik asit %38.54, takiben palmitik asit (%20.82), sonra palmitoleik asit (%12.71), linoleik asit (%12.01), stearik asit (%4.4%), palmitoleik asit olarak gözlemlendi. Diğer yağ asitleri düşük oranlarda idiler.

1.INTRODUCTION

Carotenoids are amongthe most widespread and important pigments of the various classes of pigments in nature.Carotenoids are important natural pigments, displaying yellow, orange, and red color, found widely in microorganisms and plants. Industrially carotenoid pigments such as β-carotene and astaxanthin are used as natural food colorants or feed additives in aquaculture. Several studies have shown that carotenoids combat various types of cancer and other diseases because of their antioxidant and/or provitamin A potential.Carotenoids are found in the chloroplasts and chromoplasts of plants and some other photosynthetic organisms, including some bacteria and some fungi.

Among microorganisms, some bacteria, yeasts, fungi, andalgae are well known to accumulate carotenoids as intracellular pigments (Goodwin, 1980; Johnson and Schroeder, 1995). Among yeasts, the species such as *Phaffia rhodozymais*, Genera*Rhodotorula*and *Sporobolomyces*, as well as their teleomorphs *Rhodosporidium* and *Sporidiobolus*, have beenknown for a long time to be able to produce carotenoids (Johnson and Schroeder, 1995). Also many strategies were employed to increase product yields from microorganisms, which include the use of overproducing strains, addition of bacterial and, fungal enzymes that disrupt the yeast cell wall and the development of low cost culture media that diminishes production cost (Haard, 1988; Fang, 2002). The increasing interest in microbial sources of carotenoids is related to consumer preferences for natural additives and the potential cost effectiveness of producing carotenoids via microbial biotechnology.

Rhodosporidium yeasts produce, additionally, carotenoid pigments like β-carotene, torulene and torularhodin, the precursor of which is considered to be acetyl-CoA (via the mevalonate pathway). Oleaginous yeast *Rhodosporidium toruloides* can accumulate triacylglycerols as cellular storage lipid up to 70% of the cell mass by assimilating carbohydrates from lignocellulosic biomass or othercheap materials as

the feedstock (Li, 2007; Zhao, 2010), Besides, *R. toruloides* has already been proved to be capable to grow on a broad range of substrates including but not limited to: sugars like glucose and xylose, lignocellulosic hydrolysate, and excesssludge hydrolysate (Zhao, 2007; Buzzini, 2007; Xu, 2011, Wang, 2012; Marilyn,2012). Furthermore, it can simultaneously produce carotenoids as by-products, which may have some potential values (Buzzini, 2007; Xu, 2012). The total carotenoids was reported, when glucose was used as carbon source, at 122 μg/g dry mass while the main carotenoids produced were identified as torulene, torularhodin, ɣ carotene, and β carotene (Buzzini, 2007). In order to improve the yield of carotenoid pigments and subsequently decrease the cost of this biotechnological process, diverse processes have been performed by optimizing the culture conditions including nutritional factorsespecially by response surface methodology (RSM) which is a very useful tool for optimizing process parameters. They can provide statistical models that help to elucidate the interactions among parameters at varying levels and calculate the optimal level of each parameter for a given response (Heo, 2009).

In this work, Two methods of carotenoid extraction, ultrasonic assisting and HCL assisting were carried out in carotenoids extraction from *Rhodosporidium toruloides* Y27012 compared with the control one.

Production of lipid and carotenoids by cultivating *Rhodosporidium toruloides* Y27012 was attempted. Firstly, a suitable nitrogen source , carbon sourse, the effect of C/N ratios, and some additives. Then The effects of the carbon source, nitrogen source, and additive concentrations on the production of lipids and carotenoids were simultaneously investigated using RSM. After RSM optimization fatty acids composition in the FAME was analysed by GC.

2. LITERATURE REVIEW

2.1 Carotenoids

Carotenoids are naturally occurring lipid-soluble pigments, the majority being C40 terpenoids. They act as membrane-protective antioxidants that efficiently scavenge $^1O2$ and peroxyl radicals; and besides their antioxidative efficiency is apparently related to their structure (Goodwin, 1988). The most significant part in the molecule is the conjugated double bond system that determines their colour and biological action (Sandmann, 2001). Carotenoidsare important natural pigments, displaying yellow, orange, and red color, found widely in microorganisms and plants and thesepigments also occur universally in photosynthetic systems of higher plants, algae and phototrophic bacteria. In non-photosynthetic organisms, carotenoids are important in protecting against photooxidative damage. In plants or microorganisms, carotenoids can be found as hydrocarbons (carotene; e.g., lycopene, α-carotene, and β-carotene) or their oxygenated derivatives (xanthophylls; e.g., lutein, α-cryptoxanthin and β-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin). The difference between produced amount of some carotenoids from plant and microbial ones are given in Table 2.1 (Armstrong, 1994).

Table 2.1 :compressionbetween carotenoids yields of natural and microbial sources

Caroteniods	**Natural Sources**	**content**	**Microbial Sources**	**Yield**
Astaxanthin	Krill	120 mg/kg	*Xanthophyllomyces dendrorhous*	1,080µg/g
	Arctic Shrimp	1.2 mg/kg	*Haematococcus pluvialis*	350 mg/L
β-Carotene	Carrots	183 µg/kg	*Blakeslea trispora*	420 µg/g
	Mango	131 µg/kg	*Dunaliella salina*	10,3mg/L
Lutein	Corn	12,720 µg/kg	*Chlorella zofingiensis*	21 µg/mL
	Marigold	0,3 g/kg	*Chlorella protothecoides*	225µg/mL
Zeaxanthin	Corn	5,280 µg/kg	*Dunaliella salina*	6 mg/g
	Collard	2,660 µg/kg	*Phormidium Laminosum*	5.9 mg/g

Several studies have been conducted highlighting the importance of carotenoids in healthcare as well as in nutraceutical and food industries(Bhosale, 2005; Heo,2010).

Of the 600 naturally occurring carotenoids, only a few have proven useful in human- and animal-based industries, and these have primarily received focus on their abilities to act as antioxidants

Industrially, carotenoids are used in pharmaceuticals, nutraceuticals, and animal feed additives and alsoused as colorants in cosmetics and foods. Interest in carotenoids has increased in recent years because of their beneficial effects on human health.

Carotenoids are considered as protective against cancer, coronary artery disease, and ocular diseases. Lycopene intake is inversely related to prostate cancer risk intake of foods rich in carotenoids is associated with reduced risk of cardiovascular disease; increased plasma levels of α-carotene, β-carotene, and lycopene tend to be inversely related with ischemic stroke and increased plasma levels of lutein and zeaxanthin are associated with decreased risk of age-related macular degeneration (Giovannucci, 1995;Mayne, 1996; Hak, 2004; Anon , 1993).

The carotenoids market is expected to increase to $919 million by 2015 with an annual growth rate of 2.3% (Ulirch, 2008). Most of these carotenoids are obtianed from synthetic sources; however, synthetic pigments have been perceived to cause hazardous effects to human health at high dose ranges and have been subsequently warned by the Food and Drug Administration (Kalui, 1981).

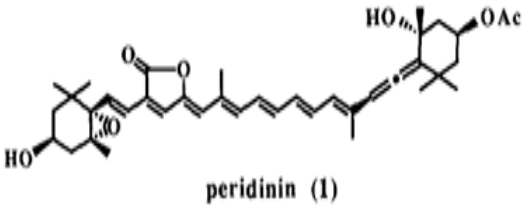
Therefore, attention is paid to investigating of suitable natural methods for its production. The potential of microorganisms that are able to convert various substrates into carotenoid pigments is gaining interest even this approach is restricted by a number of useful species and also the carotenoid yield cannot compete with chemical synthesis (Misawa, 1997).

Currently, β-carotene and astaxanthin are industrially produced from microbial sources and are widely used in food and feed industries (Bhosale, 2005; Jacokson, 2000;Lorenz, 2000).

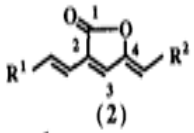
The major limitation on the use of microbial systems for commercial production is the low yield, slow growth, and high production cost compared with chemical synthesis.

2.1.1. Industrial Production: Synthesis

The first synthesis of β-carotene was reported by Karrer and Eugster (Karrer1950)and Inhoffen and co-workers (Inhoffen and Milas, 1950). The Inhoffen synthesis was later developed into an industrial process and since 1954, β-carotene has been produced commercially. The various synthesis of carotenoids employ some key reactions for the formation of the carbon-carbon double bond, in particular, the aldol condensation, the Wittig condensation, the Emmons-Horner reaction, the Julia's method, and the addition of acetylides. the total synthesis was accomplished of peridinin (Fig 2.2), which is representative of the butenolide carotenoids and is known as an auxiliary light-harvesting pigment for photosynthesis. Peridinin is a unique C_{37}-tricyclic carotenoid containing a 4-alkylidenebntenolide structure carrying an allene function in the main polyene chain (Strain, 1976). In this total synthesis, three reactions are included: formation of a 4 alkylidenebntenolide, displaying extended conjugation at the C-2 position, then synthesis of a C_{22}-allenic sulfone possessing the abnormal arrangement of an in-chain methyl group, and lastly palladium-catalyzed olefination of the vinyl triflate.



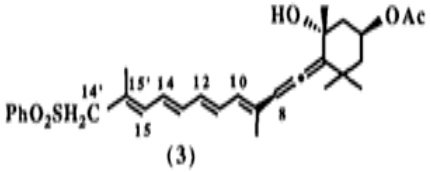
peridinin (1)



(2)

R^1=alkyl or polyene chain

R^2=polyene chain



(3)

Figure 2.1 : Structures of peridinin(1), 4- alkylidenebutenolides(2), and the C_{22}-allenic sulfone

2.1.2 Microbial Production of Carotenoids

In recent years, the interest in production of natural carotenoids by microbial fermentation has been increased. Carotenogenic microbes such as *Dunaliella salina, Xanthophyllomyces dendrorhous, Haematococcus pluvialis,* and *Blakeslea trisporahave* been investigated for large-scale production (Jacobson, 2000; Mehta, 2003; Raja, 2007). Carotenoid biosynthesis is a specific feature of the *Rhodotorula* species such as *Rhodosporidium* and and *Phaffia* genera (Kvasnikova, 1978; Martin, 1993; Meyer, 1994; Buzzini, 2001)

The pigmented yeasts have an advantage over algae, fungi, and bacteria due to unicellular and relatively high growth rate with utilizing low-cost fermentation media (Malisorn, 2008). In litreature, the typical concentrations for carotenoids produced from red yeasts were ranged from 50–350 µg/g dry weight (Davoli, 2004). Costa (1989) studied the production of carotenoids by *Rhodotorula*and reported that it produced 630 µg/g dry cells. Frengova(1994) reported that a mixed culture of *R. glutinis* and *Lactobacillus* in whey filtrate was able to produce 268 µg/g dry cells. Sucrose and glucose are the most common carbon sources used in the production of carotenoids. The use of glucose can lead to a higher efficiency in the specific production of carotenoids by *Rhodotorula spp*. (Buzzini, 2000). A number of studies have been carried out in recent years on the fermentation of agricultural wastes to produce carotenoids by different strains in shake flask fermentation that can be seen in Table 2.2 (Martin, 1993; Frengora, 1994; Bhosale, 2001).

However, the types of carotenoids and their relative amount may vary depending on the cultivation medium, temperature, metal ions, light, rate of aeration and the presence some additives (Simpson, 1964; Ausich, 1997; Wang, 2001; Flores, 2001; An, 2001; Aksu, 2005; Buzzini, 2005). Carotenoids production has been also performed successfully in non-carotenogenic microbes, such as *Escherichia coli, Saccharomyces cerevisiae, Candida utilis,* and *Zymomonas mobilis*, by transforming them with carotenoid genes from carotenogenic microbes (Misawa and Shimadaet, 1998).

*E. Coli*is a suitable host for carotenoid production because it has a powerful genetic tool system for metabolic engineering (Misawa, 1990; Ruther, 1997).

Table 2.2 : The use of agro-industrial wastes in carotenoids production by various yeasts

Substrate	Yeast	Yield	Authors
Whey	*R.glutinis*	46 mg/L of β-carotene	Marova (2011)
Potato medium	*R.mucilaginos*a	56 mg/L of β-carotene	Marova (2011)
Crude glycerol	*R.glutinis*	135.2 mg/L of carotenoides	Saenge (2011)
Whey ultrafiltrate	*R.acheniorum*	262 mg/L of β-carotene	Nasrabadi and Razavi (2012)
Whey	*S.salmonicolor*	590.4 mg/L of carotenoides	Valduga (2009)
Fermented radish brine	*R.glutinis*	19 µg/L of β-carotene	Malisorn (2009)

Carotenoid-synthesizing yeast is marketed in inactivated dried yeast from *P. rhodozymaand Phaffia Astaxanthin* by Partners Ltd`s (USA). Also spray dried *P. rhodozyma* is produced by Archer Daniels Midland (USA) as a natural source of astaxanthin, protein, and other nutrients, and utilized in many countries like European Union, Canada, USA as an ingredient in salmonids feed.

2.1.3 Microbial Carotenoid Biosynthesis

In order to discuss the response to stimulants of carotenoidproduction, it is essential to briefly describe carotenoidbiosynthesis, since the activity and quantity of thebiosynthetic enzymes are known to significantly influencestimulant activity.Acetyl-CoA is the key precursor of carotenoid biosynthesis in microorganisms. Simpson (1964) and later Goodwin (1980, 1993) reviewed the general pathways for carotenoid synthesis and concluded that carotenoid bio-synthetic pathways commonly involve three steps, these include:

1- The conversion of acetyl-CoA to 3-hydroxy-3-methyl glutaryl COA (HMG-COA) is catalyzed by HMG-CoA synthase. HMG-CoA is then converted into a C6 compound, mevalonic acid (MVA). MVA is converted into isopentyl pyrophosphate (IPP) by a series of reactions involving phosphorylation by MVA kinase followed by decarboxylation.

2- IPP is isomerized to dimethylallyl pyrophosphate (DMAPP) with the sequential addition of three IPP molecules to DMAPP. These reactions are catalyzed by prenyl transferase to yield the C20 compound geranyl geranyl pyrophosphate (GGPP). Production of DMAPP form Acetyl-CoA is represented in Figure (2.2).

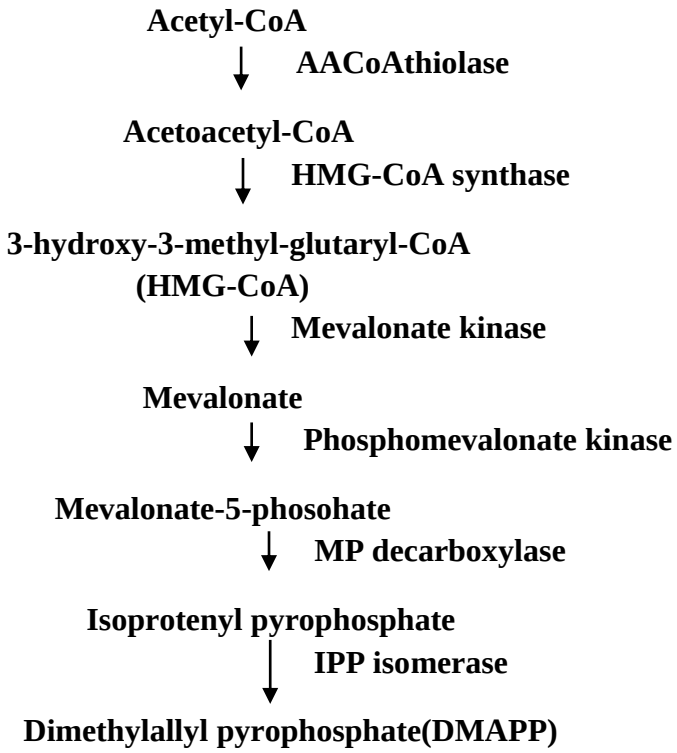


Figure 2.2: First and second steps of carotenoids synthesis(Goodwin 1980, 1993)

3- Two molecules of GGPP condense head to form phytoene, which undergoes desaturation to form lycopene and Figure 2.3 represents the schematics of microbial carotenogenesis.

As lycopene is an all-trans compound, the isomerization of the first or second double-bond of the phytoene must occur at the same stage in the desaturation process (Goodwin, 1993).

Lycopene acts as precursor of cyclic carotenoids and undergoes a number of metabolic Xanthophyllsare oxygenated products of α- and β-carotenes. Hydroxygroups are introduced at the 3 and 3′ positions of theionone rings, and epoxy groups are subsequently formedat the 5,6 and 5,6′ position as in figure 2.3.

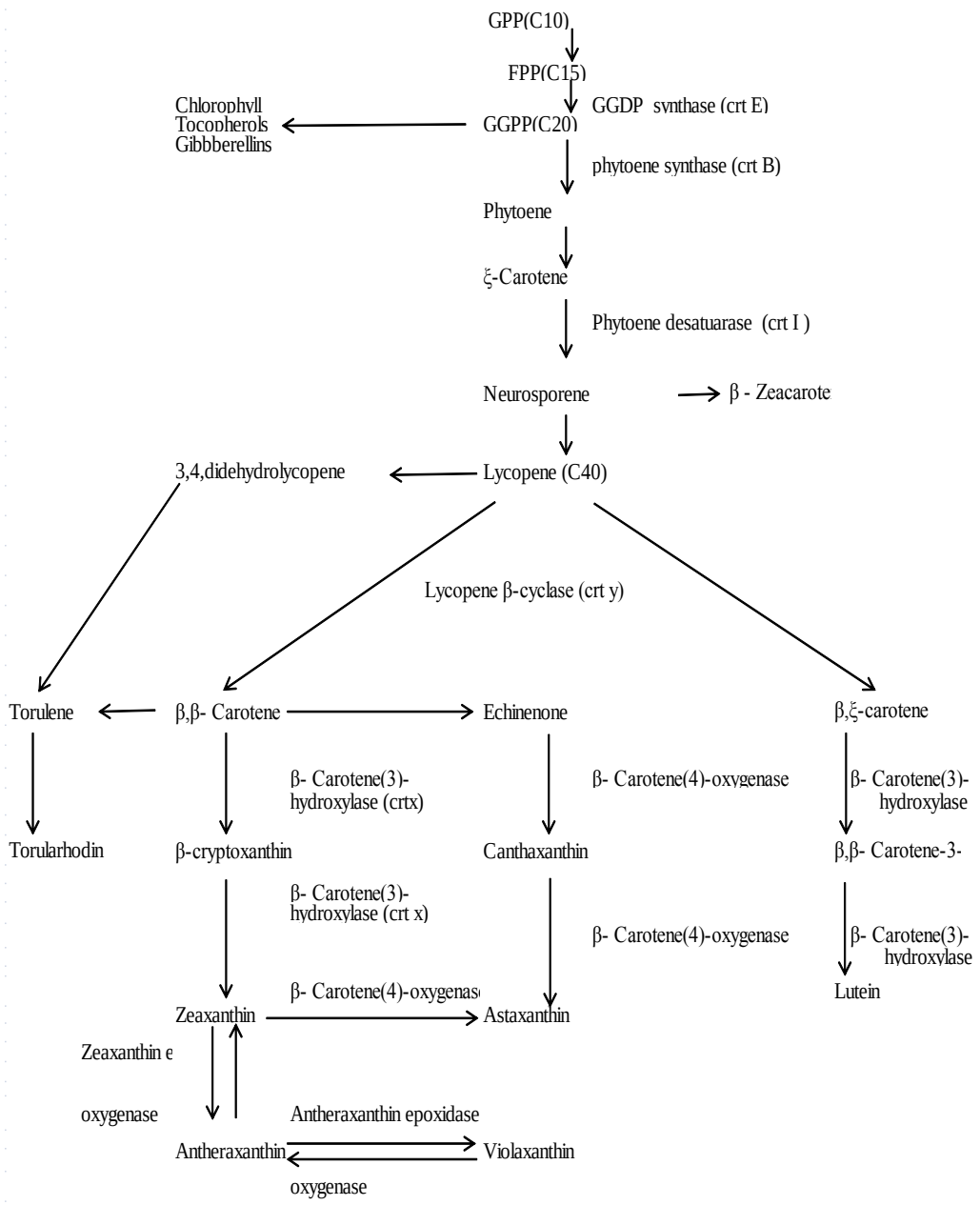


Figure 2.3: Carotenoids biochemical pathway (Sandmann, 1994)

2.1.4 Factors Effecting Carotenoids Production

2.1.4.1 Temperature

Temperature is one of the most important environmental factors affecting the growth and development of living organisms. Temperature was reported to control the concentration of enzymesinvolved in carotenoid production, and changes in enzyme concentration ultimately control carotenoid levels in microorganisms (Hayman, 1974).

The study of the biosynthetic pattern ofcarotenoid formation by *R. glutinis48-23T* cultivated at 5and 25°C in glucose medium proved that, at 5°C the synthesized carotenoids are represented mainly by β-carotene (64%) and significantly less by torulene and torularhodin, when at 25°C the pigments such as β-carotene, torularhodin and torulene were produced in concentrations of about 30% of the total carotenoids (Simpson, 1964). The reason which Bhosale introduced for increased β-carotene accumulation at 90% of total carotenoid by mutant *R. glutinis*at 20°C and a decrease at 71% of total carotenoidas the incubation temperature was increased to 30°C is that ɣ-carotene acts as the branch point of carotenoid synthesis. And also dehydrogenation and decarboxylation leading totorulene synthesis is known to be temperature dependentsince the respective enzymes are less active at lower temperature compared with the activity of β-carotene synthetase (Bhosale, 2002). *Phaffia rhodozyma*cultivated at 20°C synthesized carotenoids with prevailing astaxanthin content (85%) andsmall β-carotene (10%). But at 30°C the synthesized carotenoids were torularhodin (60%), torulene(30%) and β-carotene (5%) (Polulyakh, 1991). Buzzini and Martini (1999) alsoreported that the lower temperatures (25°C) seemed tofavor synthesis of β-carotene and torulene, whereas highertemperatures (35°C) positively influenced torularhodin synthesis by *R. glutinis.*

Xanthophyllomycesdendrorhous displayed a 50% increase in total carotenoids at low temperatures. Manipulation of the cultures in the presenceof diphenylamine and nicotine at 4°C was reported tobring about interconversion of β-carotene to astaxanthin(Ducrey Sanpietro and Kula, 1998).

2.1.4.2 Light

Carotenoid production and accumulation are reported tobe positively affected by white-light irradiation in algae, fungi, and bacteria. Thereare two aspects to the theory of photo induction. The first theory isthat light has an effect on growth of the microorganism by its important role in establishing the authentic role of white-light illumination as a stimulant of carotenoid production.

The second aspect thatincreases in the cellular accumulation (mg/g) of carotenoids are associated with increased activity of enzymes involvedin carotenoid biosynthesis.(Ausich, 1997)

Tada and Shiroishi (1982) reported that the carotenoids content cells of *R. minutais* that had been cultured at 0°C did not increase evenwith illumination. These results indicate that the carotenoid production in response to light is related to biochemicalreactions dependent on nutrient and temperature, but not to changes in precursors due to a photochemical reaction. The control mechanism ofcarotenoid production by light in *R. minutais* similar to thatof photoinduced carotenogenesis in fungi and bacteria (Tada and Shiroishi, 1982). Sakaki (2001) reported that torularhodin production of *R. glutinis*can be increased at a cost of growth following exposure to weak white light, andβ-carotenewas also increased by light irradiation, but the increase wasonly 14%. However, the negative effect on growth of theyeast can be overcome by manipulating growth conditions,as reported by Bhosale and Gadre (2002), where exposure ofa β-carotene-producing mutant of *R. glut*inisto white lightin the late exponential growth phase resulted in a 58%increase in β-carotene production with a concurrentdecrease in torulene. There are also some investigations showing that, varying light intensitiesin the presence of organic and inorganic chemicalstimulators played an important role in improvement ofcarotenoid yield from*Dunaliellasp*.

In one of these studies, β-carotene accumulation was increased when the light intensity was increasedfrom 50 to 1250 μmol photon $m^{-2}s^{-1}$gradually duringgrowth of *Dunaliella salina* (Orset and Young, 1999). However, continuous illumination was found to be most favorable for astaxanthinformation *by Rhodotorula.Haematococcus pluvialis*showed a remarkableincrease in the concentration of astaxanthin with anincrease in light intensity from 50 to 400 μmol photon $m^{-2}s^{-1}$ (Boussiba, 1992).

Lutein accumulation in*Muriellopsis sp.*was enhancedby about 40% when photon flux density increased from184 to 460 μmol photon $m^{-2}s^{-1}$. However, furtherincreases in light intensity led to a decrease in accumulation (Del Campo, 1999).

2.1.4.3 Aeration

Carotenogenesis is an aerobic process and the air flow ratein the yeast culture is an essential factor to assimilate thesubstrate as well as for growth rate, cell mass and carotenoid synthesis.

The effect of aeration is dependent on thespecies of the microorganism. The aeration of the mixed culture (*R. rubra+L.casei*) influenced not only the amountof carotenoids produced, but also the composition of individual pigments making up the total carotenoids. Increasing the air flow rate causes the relative proportion of β-carotene increased from 42.0 to 60.0%, the proportion of torularhodin decreased from 44.0 to 29.0%, while the proportion of torulene changed only slightly (9.5–11.0%) (Sinova, 2003). *R. lactosa*, shows slight increase by increasing the air flow rate.The relative proportion of β-carotene and toruleneincreased from 18.2 to 21.5% and from 10.3 to 14.6%,respectively, whereas the proportion of torularhodin decreased from 71.5 to 63.9%(Zalashko, 1990).

The optimal values of air flow rate and agitation are in the range 0.5–1.9 L/min and at 180–900 rpm for carotenogenesis by *Rhodotorula* and *Phaffia*(Frengova, 1994; Bhosale, 2001; Hu, 2006). Sakaki (1999) reported that thedissolved oxygen causes increase incontent of torularhodin produced by *R. glutinis*.

2.1.4.4 C/N ratio

Numerous previous studies have shown that the C/N ratio has a significant influence on cell growth andcarotenoid biosynthesis in some microorganisms including yeasts. Most studies have suggested that a high C/N ratio is more favorablefor the biosynthesis of carotenoids .

A study by Somashekar and Joseph (2000) found that a medium with high C/N= 16:1 ratio tended to produce lipids and carotenoids withhigh biomass which is observed at C/N=10:1. Yamane (1997) proposed that a high initial C/N ratio may decrease the consumption of NADPH for primary metabolism such as protein synthesis, so as to leave more NADPH available for carotenoids (astaxanthin) biosynthesis.

Aksu and Eren (2007) reported an optimumcarotenoid production from minimalsalts media with three different C/N ratios by theyeast *R. gracilis*, and found that a C/N ratio of 10 favoredmaximum carotenoid production. Braunwald (2013) also observed that at low initial ammonium contents (C/N: 120), the carotenoid production decreased compared to medium ammonium contents (C/N: 70).

2.1.4.5 Additives

Some agents, such as detergent additives, oils, surfactants have been known to increase carotenoids productivity.

For example,the supplementation of ethanol at 10 g/L or acetic acid at 5 g/L was reported to stimulate cell mass accumulation andastaxanthin formation in fed-batch culture of *P. Rhodozyma.*As a result, the astaxanthin concentrations of 45.62 mg/Land43.87 mg/L were obtained, respectively, which were about25% higher than control experiments which were conducted without ethanol or acetic acid (Kim, 2004).

Addition ofethanol (2%, v/v) was reported to stimulateβ-carotene andtorulene formation in *Rhodotorula glutinis* (Margalith and Meydav, 1968).

Stabnikova (1979) described the use ofethanol (3.6%) as the sole source of carbon and energy in aculture medium supporting growth and β-carotene formation by *Rhodotorula glutinis*. Gu (1997) also reported increased carotenoid production from1.65 mg carotenoids/g cells to 2.65 mg carotenoids/g cells upon addition of 0.2% (v/v) ethanol to cultures of the yeast*X. dendrrohous*. Many studies revealed that ethanol activates oxidative metabolism with induction of HMG-CoAreductase, which in turn enhances carotenoid production.

The β-carotene content in cells of *R. glutinis*increased upto 35% when phenol was added to culture medium at 500 ppm (Kim, 2004).

Flores-Cotera (2001) reported thatsupplementation of citrate in the medium at levels of 28 µM or higher notably increased the final carotenoidconcentration from 3.2 to 4.5 mg/L in cells *P. Rhodozyma.*Penicillin exerts a stimulating effect duringearly stages of the isoprene biosynthetic pathway sincemevalonate kinase activity was almost double in thepresence of penicillin (Desai and Modi, 1977).

Different sources such as oils have been investigated for production of carotenoids. Aksu and Eren (2005) reported that the supplementation ofcotton seed oil in the culture medium for growth of *R. mucilaginosa*resulted in an increased production of totalcarotenoids. The yeast produced 57.6 mg/L carotenoid withcotton seed oil, while 39.5 mg/L carotenoid was formed without the activators.

Effect of cotton seed oil and Tween 80 (1%v/v) on carotenoid production was studied with two different concentration of glucose (5 g/L, 15 g/L), and at low glucose concentrations, cotton oil significantly increased production of total carotenoids while Tween 80 gave the highest amount of carotenoids at 15 g/L of glucose concentration of 69.0 mg/L (Aksu, 2007).

The carotenoids yields in the presence of tomato juice at 2.6 ml/l, groundnut oil at 1.0 ml/l,vitamin B_2 at 3.5 ml/l or vitamin B_1 at 2.2 ml/l in a fermentation medium for growth, *Rhodotorula strain* were 34.36,17.28,11.27 and 8.3% higher than that in the control culture, respectively (Wang, 2001).

2.1.5 Types of Microbial Carotenoids

2.1.5.1 Astaxanthin

Astaxanthin is the most commonly occurring red carotenoid in microorganisms.

Two major microorganisms such as microalga *H.pluvialis*and heterobasidiomycetous yeast *Xanthophyllomyces dendrorhous*produce astaxanthin commercially (Johnson, 2003; Bhosale, 2005).Chemical structure of astaxanthin is given in Figure (2.4).

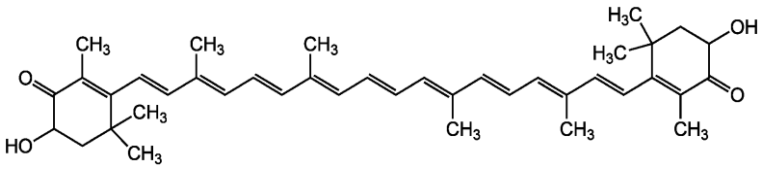


Figure 2.4 : Chemical structure of Astaxanthin(Nelis, 1991)

De la Fuente (2010) reported an improved semi-industrial process for astaxanthin production by the fermentation of *X. Dendrorhous*, in which volumetric yield was 350 mg/L of astaxanthin.

Many low cost by-products and residues of agro-industrial origin have shown the possibility of astaxanthin production from several materials such as molasses and grape juice (Haard, 1988; Breitenbach, 2001).

2.1.5.2 β-Carotene

β-Carotene is an important compound because of its role as an antioxidant, and as precursor of vitamin A in food and feed products (Borowitzka, 1992). Chemical Chemical structure of β-Carotene is given in Figure 2.5.

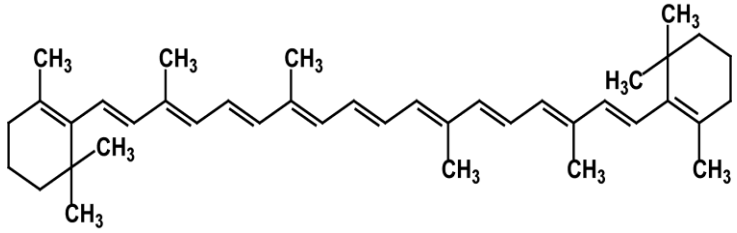


Figure 2.5: Chemical structure of β-Carotene (Borowitzka, 1992)

Commercially available β-carotene is produced mainly from the genus *Dunaliella* (Raja, 2007; Ye, 2008). Since 1980, *Dunaliella* powder and extracts have been available in Israel, China, USA, Australia, and Mexico (Johson, 1996; Borowitzka ,1999). Besides *Dunaliella*, the greatest yields have been obtained by *Blakeslea trispora* (Ciegler, 1965).Yoon (2009) reported a novel approach by the combinatorial expression of the whole bacterial mevalonate pathway for the production of β-carotene in *Escherichia coli*. The recombinant *E. coli* DH5,harboring the whole MVA pathway and β-carotene synthesis genes produced a β-carotene yield of 465 mg/L at a glycerol concentration of 2%w/v.

2.1.5.3 Lycopene

Lycopene is the most effective singlet oxygen quencher because it contains nine or more conjugated double bonds as shown in Figure 2.5 (Di, 1991).

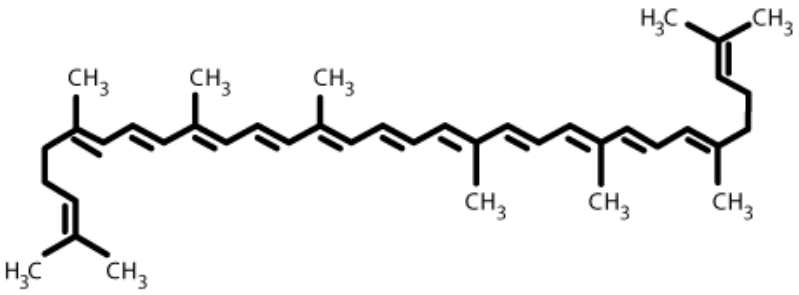


Figure 2.6 : Chemical structure of lycopene (Di, 1991).

Fungi of the genera *Phycomyces* and *Blakesleaare* are the potential lycopene producers. Chemical stimulators such as pyridine,imidazole, and methylheptenone have been reported to stimulate lycopene accumulationin*B. Trispora*and*P. blakesleeanus* (Feofiva, 1995).

Lycopene finds applications in beverages, dairy foods,confectionery, soups, nutritional bars, breakfast cereals, pastas, chips, sauces, snacks, dips, and spreads (Anon, 1992).

2.1.5.4. Lutein

Lutein is one of the fastest growing carotenoids on the market. Currently, the natural commercial source of lutein is the solvent extract of marigold (Khachik, 2005).Chemical structre is shown in Figure 2.7.

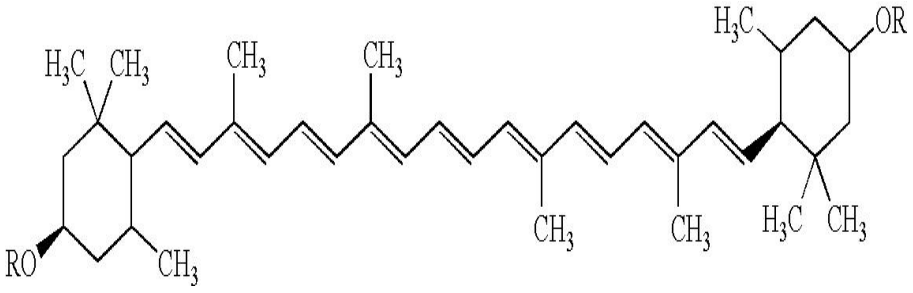


Figure 2.7 : Chemical structure of lutein (Nelis, 1991)

In recent years, several microalgae have been studied as potentiallutein sources, such as *Chlamydomonas reinhardtiiMuriellopsis sp* .and *Scenedesmus almeriensis;*however, microbial sources still lack commercial potential mainly due to lack of studies involving strain improvement and high-volume bioreactors (Francis, 1975; Del, 2001).

2.1.5.5 Zeaxanthin

Zeaxanthin is an isomer of lutein, chemical structure is shown in Figure 2.8 (Khachik, 1989; Nelis, 1991).

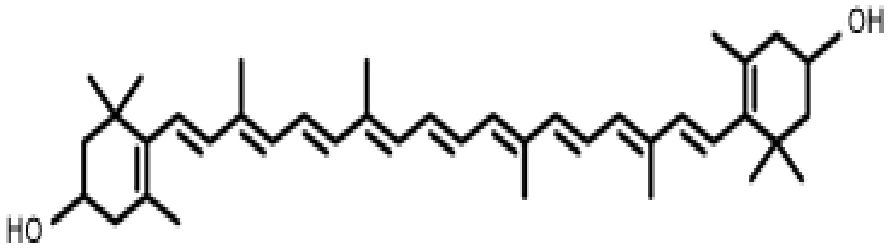


Figure 2.8: Chemical structure of zeaxanthin (Nelis, 1991)

Marine bacterium *Flavobacterium* species are well documented for their zeaxanthin production. Zeaxanthin occurs in cyanobacteria, unlike lutein, which is typically present in photosynthetic microorganismsand also in some non-photosynthetic bacteria (Fresnedo, 1991; Nelies, 1991). Other microbial sources include *Dunaliella sp*., which produces zeaxanthin under various stress and gene manipulation conditions and *Microcystis aeruginosa* (Jin, 2003; Chem, 2005).

2.1.6 Purification and Separation of Carotenoides

2.1.6.1 Extraction of Carotenoids

All classes of carotenoids are lipophilic compounds and are soluble in oils and organic solvents.

Generally, water-miscible solvents including acetone, ethanol, methanol, or mixtures of these polar solvents and other more nonpolar solvents such as hexane, tetrahydrofuran (THF), diethyl ether, and methylene chloride have been used in many studies to extract carotenoids in foods.Methanol (MeOH) alone or a mixture of MeOH with other more nonpolar solvents is also widely used (Hart, 1992).

Recently cell disintegration is achieved to increase carotenoids extraction. Generally cell disintegration is done by mechanical breakage and acid or alkalinehydrolysis (Gu, 2007). Enzymatic digestion of cells also appears as an attractive option, presumably resulting in higher recovery rates of carotenoids from the microbial matrix (Michelon, 2012).

A combination of lysozyme/lyticase with synergistic sonication and freeze thawing provide a beneficial and robust alternative to other mechanical disruption methods (Kaiser, 2007). Lipases have been used to disintegrate lipid droplets containing carotenoids and to prevent capillary plugging of the HPLC system (Johnson, 1991; Davoli, 2003). Cell distruption techniques used in carotenoid extraction are summarized in Table 2.3.

Table 2.3: Different techniques of cell disruption used in carotenoid extraction

Disruption technology	Yeast	Yield µg/g biomass	Authors
HCL and acetone	*Rhodobacter sphaeroides*	4790	Gu (2007)
DMSO, acetone and petroleum ether	*Rhodotorula graminis*	803.2	Buzzini (2001)
Freezing and DMSO	*Phaffia rhodozyma*	155.72	Michelonet (2012)
Enzymetic lysis and ultrasonic waves	*Phaffia rhodozyma*	163.12	Michelonet (2012)

2.1.6.2 Spectrophotometric Methods

The total carotenoid content was determined by colorimetry, which was performed by subjective comparison of colors or by objective measurements of color intensities with a colorimeter or photometer. Nowadays, spectrophotometry, which allows one to measure the absorption at a single wavelengths, is predominant.

During quantitavie determination of total carotenoids, the sampleis dissolved in an accurately known volume of the appropriate solvent and a 1 cm light path cuvette is filled with the solution; the matching reference cuvette is filled with the pure solvent. For quantitative determination by measuring absorbance in the UV region, matched quartz cuvettes must be used, and the solvent must be free from UV absorbing materials. The absorbanceof the solution is then determined at the appropriate wavelength. The extinction(E_{max}) of a known volume of a carotenoid solution ismeasured at the wavelength of maximal absorbance;the amount of carotenoid (C) dissolved in a givenvolume (V) is obtained from the expression:

$$C = \frac{\text{Emax.V}}{\text{E}_1\%,1\text{cm}} . 100 \quad (\mu g/L) \qquad \textbf{(2.1)}$$

In the formula,E_1%is the specific extinctioncoefficient of a 1% solution in a 1 cm light pass cell.The absorption maxima of carotenoids in varioussolvents and their extinction coefficients are given in Table 2.4 (Davies, 1971). For the determination of a mixture ofcarotenoids, the measurement is carried out at awavelength of 436nm, theE1cm1% ofβ-carotene = 2505is generally used, and the results are expressed as β-carotene. The detection limit for ß-carotene and lycopene is 0.04 µg/g (Sadler, 1990).

Table 2.4 : Specific extinction coeffiecient (E^1%,1cm) of carotenoids in stated solvents

Carotenoid	$E_{1cm,1\%}$	Wave length (λ)	Solvent
Astaxanthin	1900	473	Acetone
β-carotene	2505	457	Cyclohexane
Lutein	2336	458	Benzene
Lycopene	3370	487	Benzene
Zeaxanthin	2340	452	Acetone

Aksu et al (2005) determined the carotenoid from *R.glutinis* spectrophotometrically after biomass centrifugation and addition of acetone to extract intracellular carotenids(Aksu, 2005; Aksu, 2007). Total carotenoids from *R.glutinis* after extra4ction were measured spectrophotmterically (Frenogova, 1994; Buzzini, 2007).

2.1.6.3. Thin-Layer Chromatography

Since its introduction in the late 1950s, TLC has been the most versatile and effective method for purifying carotenoids, and remains extremely useful even in laboratories with good HPLC facilities. TLC is used both for separation and purification and for partial identification of carotenoids by comparison with authentic samples.

In carotenoid analysis TLCis mainly used for preliminary examinations to give anindication of the number and variety of carotenoidspresent and to help in the selection of a suitableseparation and purification procedure for the given mixture (Davies, 1976).

The crude carotenoids extract from *R. glutinis* was chromatographed on thin layer of a mixture of silica gel G60 and calcium hydroxide (1:1w/w) using 5% benzene in petroleum ether (b.p. 80˚C - 100˚C) as developing system. The separated carotenoids were identified by their maximum absorption and also by their $R_f$values. (Davies, 1965; Stahl, 1969)

The fraction of the crude carotenoids from *R.glutinis* was done by TLC; silica gel G was used as an absorbent and two solvent benzene (95:5), petroleum ether (95:5) were used for separation of non polar and polar compoennts (Latha , 2004). Also microbial carotenoids from *R.glutinis* were chromatoghraphed using pre-coated TLC plates (silica gel 60) (Bhosale, 2001).

2.1.6.4. High-Performance Liquid Chromatography (HPLC)

The technique of HPLC has been widelyapplied to the study and analysis of carotenoids.Compared with another methods, HPLC is characterized by short analysis time, highresolution, good reproducibility, and little structuralmodification. Generally, carotenoids are identifiedthrough their chromatographic behavior or bycoelution with authentic standards. Additionally the identity of carotenoids can be confirmed by theirUV–VIS absorption spectra, which can be recorded bya stopped-flow scanning method or on-line with a photodiode array detector. For the stationary phaseboth normal-phase and reversed-phase HPLC areused, the elution being either isocratic or with a gradient; the latter is especially employed for complexextracts containing carotenoids of widely different polarities (Britton, 1992).

A modified method of HPLC analysis was performed in detection of carotenoids with analytical HPLC equipped with a C18 column (4.6mmx 250mm, 5µm).

The mobile phase was composed of acetonitrile:dichloromethane:methanol (80:10:10,v/v/v) with aflow rate of 1.0 mL/min. The column thermostat was set at 30°C. The detector was operated at 454 nm; in alinear gradient for 45 min, maintaining this proportion until the end of the run (Manowatlana, 2012). Carotenoid analysis was performed for the extracts obtianed from *R. mucilaginosa*CBS17T and *R. Glutinis* CBS20Ttype strains by reverse phase HPLC employing a LiChrospher (100RP)18 (5lm) column and a UV–Vis PerkinElmer 900 detector (set at 450 nm). The solvent systemconsisted of solvent A: acetone and B: water, 95:5 (v/v).The flow rate was 1 ml/min. (Libkind, 2006).

2.2 Lipids

Lipids are organic compounds that are insoluble in water but soluble in organic solvents. There are two general types of lipids including complex and simple lipids.The complex lipids such as triacylglycerols, can be hydrolyzed to smaller molecules. The Acyl- and phosphor-glycerols, the most common lipidsare based on structure of glycerol and fatty acids. Acylglycerols have a glycerol backbone linked to one, two and three fatty acids with ester bonding to provide mono-di- and tri-acylglycerols, respectively (Dyal and Narine, 2005). The major components in natural oils and fats are triacylglycerols. Thus, the defined terms of oil and fat are often used to meantriacylglycerols. However, the other components still exist in small amounts withinnatural fats and oils including mono and diacylglycerols, phospholipids, waxes,steroids and carotenoids (Stauffer, 1996).

2.2.1. Microbial Lipids

Many organisms synthesize lipids as anintegral part of their metabolites and as energy storage compounds.The ability of certain microorganisms to accumulate high amounts of lipids has been known for years, but only in thelast decades, real efforts have been made to unravel the under lying biochemical pathways.

In general, lipid accumulation is found in some yeast, fungi and small number of algae. In eukaryotic microorganisms, such as *Saccharomyces cerevisiae* and *Candida utilis*, the biosynthetic pathway of triacylglycerides isessential to cell vitality yet lipid content is normally <5% of their cellular biomass (Mlickova, 2004). Although all microorganisms have to synthesize minimum amount of lipid content for their membranes and other structures, only small number of microorganisms can accumulate lipid higher than 20% of their biomass. They are named as "oleaginous microorganisms" (Ratleadge, 2002).

Therefore, some yeast that accumulates high concentration of lipid is defined as "oleaginous yeast" (Thorp and Ratledge, 1972). It was reported that only 25 species in 700 yeast species can accumulate lipid more than 20% of biomass such as *Rhodotorula spp.*, *Yarrowia lipolitica*, *Lipomyces starkeyi* and *Cryptococcus curvatus*(Ratledge, 2004). The lipid accumulated in oleaginous yeasts is mainly in triacylglycerol form and equivalent in chemical composition of the vegetable oils and fats (Ratledge and Evan, 1989).

The relative amount of fatty acid compositions in oleaginous yeasts are oleic acid (18:1) > palmitic acid (16:0) > linolenic acid (18:2) = stearic acid (18:0) (Ratledge and Tan, 1990).

Fatty acid composition of lipid produced by various yeast strains growing on sugars or similarly –metabolized like glycerol, molasses, etc substrates in culture conditions favoring the accumulation of microbial lipid is explained in Table 2.5.

Table 2.5 : Composition of lipids produced by various yeast strains

Strain	**Lipid %**	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	**Reference**
Candida sp. 107	n.r.	28	n.r.	8	41	17	17	Davies (1957)
Candida sp.	40.3	23	13	3	54	5	2	Aggelis (1997)
R. toruloides	67.5	20	1	15	47	13	3	Li (2007)
R. toruloides	65.2	34	T.	13	48	1	T.	Hu (2009)
R. toruloides	62.1	26	2	5	62	3	T.	Wu (2010)
R. toruloides	55.6	43	T.	16	35	2	T.	Wu (2011)
L.starkeyi	61.5	37	4	6	49	1	T.	Zhao (2010)
Y.lipolytica	43.2	15	2	11	47	21	3	Aggelis (2002)

T:<0.5 % w/w, nr: not reported

2.2.2 The Biochemistry of Lipid Accumulation by Yeasts

It is necessary to understand how oleaginous yeasts synthesize fatty acid and accumulate lipid in their cells. In some oleaginous yeasts, the content of storage lipid may reach to 70% of their biomass when culture in optimized medium. The excess carbon is converted to lipid by lipid biosynthesis which is accumulated within lipid bodies (Ratleadge, 2004).

Non-oleaginous yeasts such as *Saccharomyces cerevisiae* and *Candida ultilis* never accumulate lipid higher than 10% of their biomass (Rattray, 1989). When cultivation of non-oleaginous yeasts in nitrogen-limiting medium they convert excess carbon source into various polysaccharides such as glucans and mannans (Ratledge and Wynn, 2002).

The first biochemical reaction after nitrogen exhaust from the medium is an activation of adenosine monophosphate deaminase which catalyzes the conversion of AMP to inosine 5'-monophosphate and ammonium (Evan and Ratledge, 1985).

$$\text{AMP} \xrightarrow{\text{adenosine monophosphate deaminase}} \text{inosine 5'-monophosphate} + NH_4^+$$

Ammonia-scavenging mechanism including decreasing in AMP concentration results in changing of oxygen consumption by the cells and releasing of CO_2 (Ratledge and Wynn, 2002), causing reduction or depletion of isocitrate dehydrogenase activity.MestThe isocitrateis not metabolized via tricarboxylic acid cycle because the depletion of isocitrate dehydrogenase activity would then equilibrate to citrate via activity of aconitase (Evan and Ratledge, 1983).

$$\text{Isocitrate} \leftrightarrow \text{Aconitate} \leftrightarrow \text{Citrate}$$

Citrate is transported out of mitochondria to cytosol by malate/citrate translocase system (Evan and Ratledge, 1985). The citrate within cytosol is cleaved byATP:citratelyase, which is not found in non-oleaginous microorganism but found in eukaryotic which can accumulate triacylglycerol lipids (Botham and Ratledge, 1979).

$$\text{Citrate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + \text{ADP} + \text{Pi}$$

Accumulation of lipids in oleaginous yeast and molds are shown in Figure 2.9 (Ratledge and Wynn,2002).

There is a direct correlation between ATP:citratelyase activity and the ability of lipid accumulation in yeasts, filamentous fungi and other oleaginous microorganism (Ratledge and Wynn, 2002). No organism has been found to accumulate lipid higher than 20% of their biomass without ATP:citratelyase activity.

However, there are a small number of yeasts that have been found this activity but do not accumulate lipid. There is no correlation between the absolute activity of ATP:citratelyase and the concentration of lipid accumulation in various yeasts and fungi. Thus, it cannot be the sole explanation for accumulation of lipid (Ratledge, 2002).

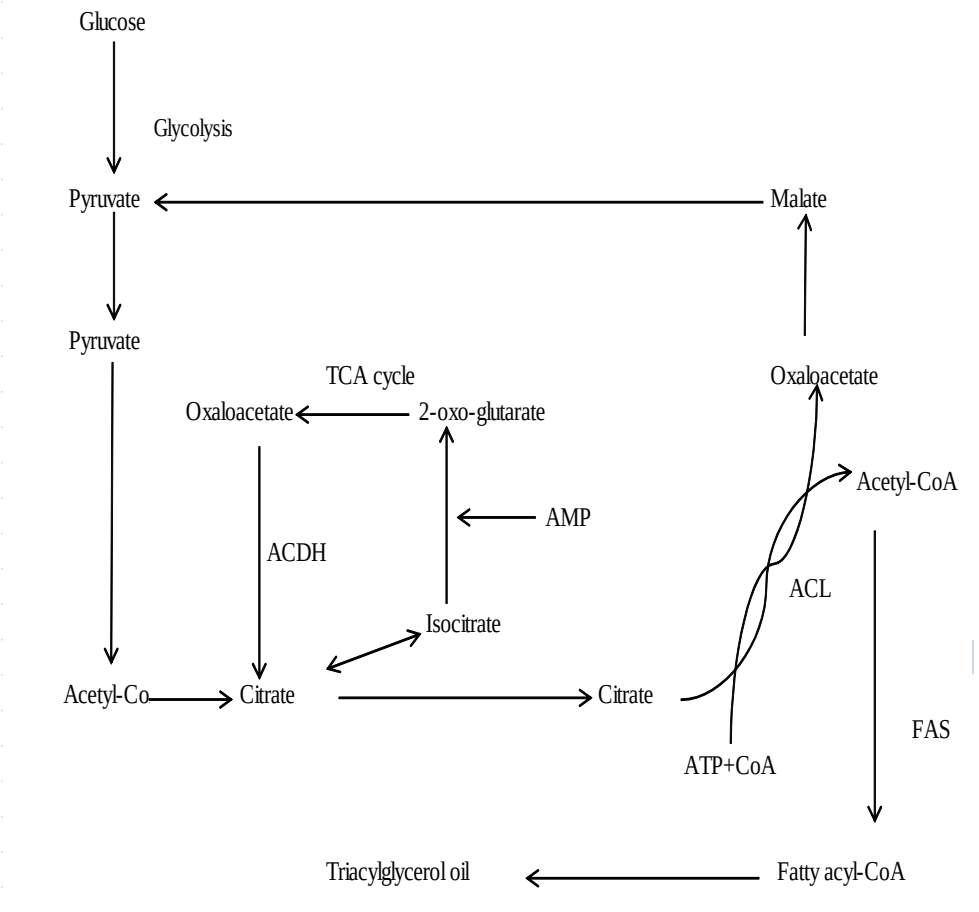


Figure 2.9: Outline of the accumulation of lipids in oleaginous yeast and mold (Ratledge and Wynn,2002).
*ICDL: isocitrate dehydrogenes; TCA cycle: tricarboxylic acid cycle;
ACL:ATP citrate lyase, FAS fatty acid synthase

2.2.3 Efficiency of Yeasts for Lipid Accumulation

The fatty acid profiles depend on oleaginous yeast types and growth conditions. Environmental conditions such as temperature, pH, substrate, C/N ratio and oxygen have influence on efficiency of yeasts for lipid accumulation. In principle, a good range of productivity of lipid product can be achieved in batch, fed-batch and continuous processes. The process of yeast lipid production was improved in both batch and single stage continuous fermentations (Gill, 1977; Evans and Ratleadge, 1984).Gill (1977) used single stage continuous process for culturing oleaginous yeast, *Candida 107*, and found that this yeast strain accumulated lipid at 35% of dry biomass when glucose was used as the substrate. Lipid production by *Candida curvatas (Cryptococcus curvatus*) was investigated both in batch and continuous fermentations using media containing glucose, sucrose, lactose, xylose or ethanol. Results showed that when xylose was used, lipid production of 49 % of dry biomass was obtained in batch fermentation while 37% of dry biomass was attended with continuous fermentation (Evans and Ratledge, 1983). Lipid production by *Rhodosporidium toruloides* Y4 in batch fermentation resulted in accumulation of lipids up to 76% of dry biomass when glucose was used as a substrate (Li, 2006). Ykema (1989) investigated lipid production by *Candida curvatus* using various culture systems using whey permeate and lipid content of 33% of dry biomass were obtained when a partial recycling method was used. Meesters (1996) used glycerol as a carbon source in fed-batch fermentation with *Candida curvatus* and obtained a cell density of 118 g/L, and lipid content was only 25% of dry biomass. Oleaginous yeast, *Rhodosporidium toruloides* Y4, was reported to produce lipid at 67.5% of dry biomass by using fed-batch mode (Li, 2007). The rate of lipid synthesis in oleaginous microorganisms depends mostly on high C:N ratio (Ratledge, 1982).*Apiotrichum curvatum* has given high lipid content such as 50% of cells dry weight with whey permeates in continuous fermentation with a high C:N ratio (Ykema, 1988). Because of the high accumulation of citrate which is transported to the cytosol for further breakdown to produce acetyl-CoA. High lipid accumulation in cells of oleaginous yeast is obtained under limiting nitrogen concentration conditions. The oleaginous yeast *L.starkeyi* delivered lipid content of 68% at a C:N ratio of 150 compared to 40% in the presence of a C:N ratio of 60 while growing on digested sewage sludge (Angerbauer, 2008).

Similar results were obtained by Patil (2010), they used a semi-synthetic medium for cultivation of *L. starkeyi*, and reported a maximum lipid content of 27% fed-batch fermentation, which is slightly lipid content of 23% in batch fermentation of *L. starkeyi* on glucose in a semi-synthetic medium with C:N molar ratio of 56.7.*Rhodotorula glutinis* with batch fermentation can accumulate lipids up to 49% of cell dry weight and14.7 g/L lipid, while production in a continuous mode of operation, lipid contents increased to 60.7% and 23.4 g/L lipid (Dai, 2007).

Lipid accumulation by*RhodosporidiumtoruloidesY4*was directly linked to the carbon to phosphorus (C:P) molar ratios of the culture media. Moreover, such lipid accumulation phenomenon was effective regardless of the presence of high amounts of nitrogensources. Thus, cellular lipid content and lipid yield were 62.2% and 0.205 g/g glucose, respectively, using a medium with a carbon to nitrogen C:N molar ratio of 6.1 and a C:P molar ratio of 9552 mol/mol. (Wu, 2010).

Sulphate limitation was effective to promote accumulating substantial amounts of intracellular lipid by the oleaginous yeast *RhodosporidiumtoruloidesY4*. When it was cultivated using a medium with an initial carbon-to-sulfur (C:S) molar ratio of 46.750cellular lipid content reached up to 58.3% (Wu, 2011).

2.3 Oleaginous Microorganism:*Rhodosporidium toruloides*

Rhodosporidium toruloides which is a non-pathogenic, pink-coloured basidiomycetous fungus, can accumulate lipids to more than 70 % of its dry cell weight (Ratledge, 2002; Li, 2007). This yeast is also a good producer of carotenoids and biotechnologically important enzymes, such as cephalosporin esterase and epoxide hydrolase (Politino, 1997; Buzzini, 2007).

More importantly, *R.toruloides*has excellent tolerance of inhibitory compounds that are found in biomass hydrolysates (Hu, 2009).

*R. toruloidesis*is considered to be an unique yeast strain of great biotechnological potential. In comparison with *Saccharomyces cerevisiae*, *R. toruloides* contains ATP:citrate lyase (ACL), and the mitochondrial *β*-oxidation (MBO) and carotenoid biosynthetic pathways. Acetyl-CoA, which results from the cleavage of citrate by ACL, is a major source for lipid synthesis although cytoplasmic acetyl-CoA synthase can produce acetyl-CoA from acetate in oleaginous fungi and eukaryotes.

Unlike β-oxidation in peroxisomes, the MBO mitochondrial β-oxidation pathway recovers more energy through the degradation of fatty acids by using the flavin adenine dinucleotide (FAD) cofactor, which couples with the respiratory chain to produce ATP.

Moreover, MBO-associated enzymes are required to metabolize branched-chain amino acids to produce acetyl-CoA (Maggio, 2004). Thus, MBO may provide alternative acetyl-CoA and energy sources for fatty acid synthesis by degrading amino acids and membrane lipids.

Two genes that code putative carotenoid synthesis-related enzymes, phytoene synthase (PSY1) and phytoene dehydrogenase (CRTI), were identified, revealing the genetic basis for the formation of the pink-coloured pigments by this organism (Zhu, 2012).

The biochemistry of lipid accumulation inmicroorganisms using glucose as the carbon sourcehas been extensively investigated. Previous dataindicated that lipid production requires media withan excess sugar or similar components anda shortage of other nutrients, usually a nitrogensource, while de novo lipid biosynthesis occurs(Boulton and Ratledge, 1983).

When hydrophobicmaterials are used as substrates, lipid accumulationis a primary anabolic activity evolving simultaneously with biomass formation (Papanikolao, 2001).

It has been shown that the activity of isocitrate dehydrogenase in oleaginous microbes is lowunder nitrogen-deficient conditions because of thediminishing return of its allosteric regulator AMP, resulting in a metabolic flux shift to lipid biosynthesis. In addition, ATP:citrate lyase and malicenzyme are also important (Ratledge and Wynn, 2002). Most eukaryotic oleaginous species are yeasts,such as *Cryptococcus sp., Lipomyces sp., Rhodotorula sp., Rhodosporidium sp.* and *Trichosporon sp*. (Ratledge, 2002). Among them, *Rhodosporidium toruloidesis*is known to accumulate high quantities of lipids andsuch feature makes it attractive for the edible foods sector and biodiesel industry (Liu and Zhao, 2007).

Despite the fact that *R. toruloides* showedan excellent capability for lipid production, the detailed molecular mechanism for lipid accumulation remains unexplored for this basidiomycotespecies.

The genome of *R. toruloides*has not been sequenced and no genetic work has been reportedfor this so far.

2.3.1 Enzymes of Lipid and Carotenoids Biosynthesis in *Rhodosporidium toruloides*

-ATP:citrate lyase (ACL) supplies the prerequisite acetyl-CoA for fatty acid biosynthesis in oleaginous yeasts (Boulton & Ratledge, 1983). There was a decrease in activity of ACL when the yeast utilized either endogenous or exogenous lipid (Jane, 1988).

-Malic enzyme occurs in the cytosol and its putative role in lipogenesis is to prove NADPH for fatty acid biosynthesis (Botham & Ratledge, 1979; Ratledge, 1986; Ratledge, 1987).

-Carnitine acetyltransferase (CAT) serves to transport acetyl units into and out of the mitochondria and peroxisomes (Fukui & Tanaka, 1979).

-Isocitrate lyase(ICL) regulates the simultaneous operation of the tricarboxylic acid and glyoxylate cycles is induced in cells grown on C_2 compounds such as ethanol or acetate, or on fatty acids, which are degraded to C_2 compounds.

-NADP -dependent isocitrate dehydrogenase (ICDH). There was a slight increase in ICDH activity during growth on exogenous lipid but there was no significant increase in activity during growth on endogenous lipid (Jane, 1988).

-Catalase is regarded as a marker enzyme for peroxisomes (Fukui & Tanaka, 1979). The changes in specific activity of the enzyme were slight during lipid utilization (Jane, 1988).

Jane(1988) had mentioned in his research the changing of the enzyme activities in two strains of *R. toruloides*and their activities in many growth medium that can be seen in Table 2.6.

The enzymes which related to carotenoides biosynthesis was mentioned in some researches and until now there weren't many researches in definition of them.

Two genes that code putative carotenoid synthesis-related enzymes, phytoene synthase (PSY1) and phytoene dehydrogenase (CRTI), were identified, revealing the genetic basis for the formation of the pink-coloured pigments by *R.Toruloides.*(Zhu, 2012).

Carotenoids biosynthesis enzymes were also mentioned in the research of Kim (1997) which containGGDP synthase (GrtE)**,** Phytoene synthase (Grt B)**,** Phytoene desatuarase (Grt Y)**,** β -Crotene 3-hydroxylase**,** β-Carotene 4-oxygenase **,** Zeaxanthin expoxidase**,** Antheraxanthin expoxidase**,** Oxygenase.

Table 2.6 : Activities of some enzymesof *R. Toruloides*strains

Yeast	Growth medium*	ACL	CAT	Catalase	ICDH	ICL	Malic enzyme
***R. toruloids* CBS 14**	**Nitrogen-limited**	65	95	4×10^{3}	45	7	37
	Carbon-starvation	11	92	2×10^{3}	40	62	20
	Triolein	8	469	2×10^{3}	64	137	21
***R. toruloids* ATCC 26217**	**Nitrogen-limited**	74	96	4×10^{3}	32	4	18
	Carbon-starvation	13	101	2×10^{3}	43	65	25
	Triolein	3	705	1×10^{3}	49	144	0

Enzyme activity {nmol(mg protein)$^{-1}$}. *Nitrogen limited =lipid accumulating:carbon starvation=endogenous lipid utilizing ;triolein=exogenous lipid utilizin (Jane, 1988)

3 MATERIAL AND METHODS

3.1 Organism and Medium

R. toruloides Y27012 was maintained at 4°C on PDY agar slant (2% glucose, 1% yeast extract, 1% peptone, and 2% agar). (Xu, 2012; Hu, 2009 , Zhou, 2013).Then the culture was sub-cultured twice a month.

3.2. Incubation

Yeast cellswerepre-cultured in the medium containing 2 % glucose, 0.1 % KH_2PO_4, 0.05 % yeast extract and 0.02 % $(NH_2)_2SO_4$ at 30°C using shaker rotating at200rpm for 24 h (Liu, 2009; Xu, 2012; Zhao, 2011). For batch fermentation 100 mL of medium which was composed of KH_2PO_4 (8 g/L), $MgSO_4.7H_2O$ (0.5 g/L), carbon source and nitrogen source was used for carotenoids and lipid production, , shaked at 200 rpm/min, and incubated at 30°C after adding 2 % v/v of the pre-culture (Liu, 2009; Xu,2012). Batch fermentations were carried out for 5 days and the pH was not adjusted during the process (Buzzini and Martini, 1999).

3.3 Harvest Method

Cells in 10 mL culture broth was washed twice with distilled water, and then dried at 80°C until constant weight to obtain DCW (dry constant weight). Cell biomass was calculated according to equation (3.1) (Economou, 2010; Huang, 2013; Wu, 2010; Hu,2009).

$$\text{Cell biomass}=x = \frac{\text{dry weight(g)}}{\text{volume (L)}} \quad \text{(Economou, 2010)} \qquad \textbf{(3.1)}$$

3.4 Metabolite Production

3.4.1 Carotenoid Production

The total carotenoid content was determined by colorimetry after extraction by aceton.

3.4.1.1 Carotenoid Extraction

Two extraction methods,ultrasonic treatment and HCl-assistedextraction for carotenoid extraction from *R.toruloides Y27012* werecompared with the control extraction method in term of efficiency (Gu, 2008).

Glucose as carbon source (40 g/L) and yeast extract as nitrogen source (3.72 g/L) were used in the medium of the batch fermentation to have carbon:nitrogen (C:N) ratio of 40.The detailed extraction process of each method was described as follows:

(1) HCl-assisted extraction procedure: The dried biomass of *R.toruloides Y27012* was soaked into 3 mol/L HCl solution at 28°C, shaking at 100 rpm for 30 min, and then centrifuged at 10,000 rpm for 20 min. The supernatant was cast off, and then acetone was added to the broken cells at 40 mL/g (liquid /solid) ratio. Thereafter, extraction of carotenoids was carried out at 100 rpm for 30 min at 30°C. Then centrifuged at 10,000 rpm for 20min (Gu, 2008; Deming, 2006; Wang, 2008).

(2) Ultrasonic extraction procedure: The dried biomass of *R. toruloides Y27012* was put into 250 mL flask, and then acetone solvent was added at 40 mL/g (liquid /solid) ratio. After ultrasonic treatment in ultrasonic crasher for 10 min at 0°C, the flask was kept in water bath of 28°C shaking at 100 rpm for 30 min in order to break the cells of yeast. The sample was centrifuged at 10,000 rpm for 20 min to obtain the supernatant containing carotenoids (Gu, 2008).

3.4.1.2 Total Carotenoid Content

The total carotenoid content (measured as β-carotene) was determined by measuring the optical density at 480 nm with the UV–Vis spectrophotometer (UV-1700 SHIMDZU). Following equation was used to determine the carotenoids yield (Gu, 2008).

$$\text{Carotenoids yield } x = \frac{1000\text{ADV}}{0.16*\text{W}} \text{ (µg/g dry biomass)} \quad \textbf{(3.2)}$$

Whereas; A is the absorption at 480 nm, D is the dilution ratio, V is the volume of acetone, 0.16 is the extinction coefficient of carotenoids, W is the weight of dry cell (g)

$$\text{Carotenoids yield } x = \frac{1000\text{ADV}_1}{0.16*\text{V}_2} \text{ (mg/L)} \quad \textbf{(3.3)}$$

Whereas; A is the absorption at 480 nm, D is the dilution ratio, V_1 is the volume of acetone added, 0.16 is the extinction coefficient of carotenoids, V_2 is the volume of fermented liquid(Deming, 2006).

3.4.2 Lipid Production

3.4.2.1 Lipid extraction

Lipid production was also investigated from *R. toruloides Y27012* . Lipid content was determined according to (Folch, 1957).

Samples of dry biomass was homogenized with glass beads and extracted with 10 mL of chloroform–methanol (2:1, v/v) for 48 h at room temperature. The resulting homogenate was filtered, washed with 10 mL of 0.9 % NaCl solution.

Then centrifuged at 2000 rpm for 5 minutes, the lower chloroform phase containing oil was obtained. The chloroform phase was evaporated under vacuum rotary evaporator and the lipid content was calculated according to the following equation (Folch, 1957):

$$\text{lipid yield} = \frac{\text{dry weight(g)}}{\text{volum (L)}} \quad \textbf{(3.4)}$$

Fatty acid compositions of the obtained lipids were determined by GC after preparation of methyl esters according to AOAC 966.33.

3.4.2.2 Fatty Acid Methyl Ester (FAME) Preparation

Methanolic NaOH was added to lipid samples and boiled for about 8 min, then 5 mL of BF3 14% was added and continued boiling for 2 min. After 5 mL of heptane was added and boiled for 1 min. The heat was removed and 15 mL of saturated NaCL solution was added and shaked for about 15 s. 1 mL of upper heptane solution was transferred into glass tubes and small amount of Na_2SO_4 was added to remove H_2O. FAMEs were kept at 4°C until GC analysis.(AOAC, 1969).

3.4.2.3 Fatty Acid Composition Analysis with Gas Chromatography

FAMEs were analyzed by capillary GC, thermoquest Tracw GC 2000 (length 30 m), to determine the fatty acid profiles. The injector and detector temperatures were 250 and 260ºC, respectively. Oven temperature was held at 150ºC for 3 min followed by increase to 215ºC with a ramping at 10ºC/min and temperature was held isothermally for 40 min. Injection volume was 1 µL whereas the carrier gas was helium at 1.1 mL/min. Average results were calculated as mol%.

3.4.3 Experimental Design

The effect of different nitrogen and carbon source ,different ratio of C:N and various additives on lipid and carotenoids yield from *R. toruloides Y27012* have been studied.

In the first part of the preliminary experiments, the effect of nitrogenous compounds yeast extract (3.72g/L), peptone (3.38g/L), ammonium sulfate (1.73g/L) were evaluated in presence of glucose (40g/L) at C:N ratio of 40:1.

Then Glucose(36.36% carbon), glycerol (contain 39% carbon), and xylose (40% carbon) were evaluated as carbon source and yeast extract (3.72) as nitrogen source.

The growth conditions for *R. toruloides Y27012* were optimized for lipids and carotenoids yields with different C:N ratio 60:1, 40:1, and 20:1 by having different glucose concentration as carbon source and a single concentration ofyeast extract.

For a part of the study, Cotton seed oil, linseed oil, Tween 80,and Tween 20, ethanol, and acetic acid were added at different concentrations to glucose and yeast extract containing growth medium at C/N=20 to examine the effect of activator on the growth, lipid, and carotenoids production (Aksu, 2007; Taoka, 2010).

3.4.4 Statistical Analysis

Regression analysis and statistical significance and response surface applications were performed by using Modde 10.1 (Umetrics, Sweden) program. Central composite design (CCD) with glucose conentration g/L (x_1), yeat extract concentration g/L (x_2), and ethanolg/L (x_3), at three levels was followed to determine the response pattern and synergy of the variable.

Compliance of the model was evaluated by results of variance analysis (ANOVA).

The quadratic response surface model was fitted to the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad \textbf{(3.5)}$$

where Y is the response, β_o is the intercept, β_i linear (first order model), β_{ii} quadratic, β_{ij} interaction regression coefficients and X_i and X_j are the independent variables.(Saenge, 2011).

4 RESULTS AND DISCUSSION

4.1 Screening for The Method of Carotenoid Extraction

Studies have been reported on extraction of carotenoids from various materials (Francisco& Octavia, 1997). But there are no reports on extraction of carotenoids from *R.toruloides Y27012* which contains abundant carotenoids. In this work two different extraction method including ultrasonic (US) assisted and HCl assisted methods were employed and compared with control method. Effect of extraction method on the maximum dried cell, total carotenoids concentration and product yield is given in Table 4.1.

Table 4.1 : Effect of extraction method on the maximum dried cell and total carotenoidsconcentration (X_m ,P_m)and product yield $Y_{p/x}$

Extraction methods	X_m g/L	P_m mg/L	$Y_{p/x}$ µg/g
HCL- assisted	35.41±1.28	31.24±0.78	977±43.25
US-assisted	38.28±0.72	15.23±2.72	394.71±73.95
Control	43.69±2.87	23.05±0.39	544.98 ±32.10

The results were expressed as mean ± SD deviations of triplicate measurements

It can be seen from Table 4.1 that, HCl-assisted extraction of carotenoids is the most effective methods with yield of 977±43.25 µg/g, whereas the lower yield of carotenoids was observed by ultrasonic treatment and control samples such as 394.71±73.95µg/g and 544.98 ±32.10 µg/g, respectively.

This might be due to to the in effective disruption of cell walls which prevented more carotenoidsdissolved from the*R. toruloides*cells getting into theacetone solution, than in HCl-assisted extraction. Gu (2008) compared three methods such as grinding, US and HCl assisted extraction on carotenoids extraction yield from *Rhodobacter sphaeroides* and as a result HCl-assisted method gave the highest yield of carotenoids such as4650 µg/g, whereas USassisted extraction gave 664 µg/g carotenoids yield.

4.2 Effect of Nitrogen Source on Carotenoids and Lipid Production

After the most effective method of extraction was chosen, the effects of different nitrogen sources such as yeast extract (3.72 g/L), peptone (3.38 g/L), and ammonium sulfate (1.73g/L)were examined on the growth, lipid, and carotenoids production.For these experiments, glucose was used as the carbon source and the C:N ratio was fixed to 40:1. Effect of nitrogen sources on the maximum dried cell, total carotenoidsconcentrationand product yield is given in Table 4.2. As can also be seen form Table 4.2, the three investigated nitrogen sources had given almost the same amount of biomass. But maximum dry biomass was obtained in medium containing yeast extract.

Table 4.2 : Effect of nitrogen sources on the maximum dried cell and total carotenoidsconcentration(X_m ,P_m)and product yield $Y_{p/x}$

Index	X_m**(g/L)**	P_m**(mg/L)**	$Y_{p/x}$ **(µg/g)**
Yeast extract	35.41±1.28	31.24±0.78	977.12±43.25
Peptone	34.39±0.61	24.48±2.39	626.08±63.21
Ammonium sulfate	34.29±1.94	11.72±2.07	334.22±57.76

The results were expressed as mean ± SDdeviations of triplicate measurements

The effects and interactions of media components on the production of β-carotene by *Rhodotorula graminis* was investigated by Ferrao and Garg (2012). Medium components such asmannitol, potassium nitrate, yeast extract, monopotassium hydrogen phosphate and magnesium sulfate were screened and results suggested that higher amounts of mannitol, yeast extract, potassium nitrate and magnesium sulfate would positively affect both biomass and β-carotene accumulation.Yeast extract was critical for both β-carotene and biomass accumulation, and as a result yeast extract gave the highest carotenoids productivity with 220 µg/L at concentration between 9.5-10 g/L according to central composite design.

It was indicated that, complex organic nitrogen sources resulted in higher growth and carotenoids accumulation as compared to inorganic sources, and yeast extract gave 13.1g/L biomass and 33.0 mg/L carotenoids production (Bhosale, 2001).Aksu (2005) used ammonium sulfate as the single nitrogen source for *Rhodotorula mucilaginosa* growth, and it had been noticed that total carotenoids production and organism growth rates increased with increasing initial ammonium sulfate concentration up to 2 g/L carotenoids concentration and yields were 63.0 mg/L, 4.2 mg/g, whereas biomass was 4.9g/L. In another research conducted by Wang and other β-carotene content was found to be 13.43 mg/L when yeast extract at 4.23g/L, glucose at 12.11g/L were used as nitrogen and carbon source for *Rhodotorula glutinis RG6*, respectively (Wang, 2007). When the effect of yeast extract, ammonium sulfate and peptone and other nitrogen were invested on the carotenoids and lipid production by *Rhodotorula glutinis*, yeast extract gave the highest biomass, lipid content and carotenoids concentrations were 6.33 g/L, 32.63%, and 129.94 mg/L, respectively (Saenge, 2011).

Yeast extract also gave the highest lipid content (44.78±5.42%)and lipid yield (15.23±2.06 g/L) when glucose was used as carbon source. Lipid yields and contents of this study is given in Table 4.3.

Table 4.3: Lipid yields and lipid contents with different nitrogen sources

Index	**Lipid, g/L**	**Lipid content, %**
Yeast extract	15.23±2.06	44.78±5.42
Peptone	9.50±0.13	27.46±0.59
Ammonium sulfate	8.16±0.91	22.30±1.28

The results were expressed as mean ± SD deviations of triplicate measurements

Among the various nitrogen sources as can be seen from Tables4.2 and 4.3 together, yeast extract was the best nitrogen source for lipid and carotenoids yieldsas 44.78% and 977.12 ± 43.25 µg/g, respectively followed by peptone and ammonium sulfate. In this research the highest biomass and lipid accumulation occursunder certain conditions, which can be considered as critical,and these conditions include nitrogen source.

In another research, cellmass, lipid content in the culture of *Rhodosporidium toruloides* 21167 reached 18.5 g/L, 63.2% (w/w), respectively, after it was grown for 144 h in the medium which contained cassava starch as carbon source, and yeast extract as nitrogen source (Wang, 2012).

Itwas found that the neutral lipids decreased during thecarbon-limited stationary phase, and increased during thenitrogen-limited batch growth of *Rhodosporidium toruloides NCYC 921*. The maximum lipid content was obtained with 34 g/L glucose and 0.5 g/L yeast extract at 24%w/w (Andrade, 2012).

4.3 Effect of Carbon Source on Carotenoids and Lipid Production

Carbon source is the most studied parameter toinfluence carotenogenesis. Metabolism of yeasts actsdepending on the kind of carbon source in themedium. Glucose and other fermentable sugars aremetabolized by the glycolytic pathway, and then analcoholic fermentation. Yeasts in general are able to utilize several different carbon sources for the production of cell mass, lipids and carotenoids.

In Table 4.4, effect of carbon sources on the maximum dried cell, total carotenoidsconcentrationand product yield is given in Table 4.4. Assimilation of different sugars as carbon source by the red yeast *Rhodosporidium toruloidesY27012*for its growth and pigmentation shows that highest carotenoids yield was 977.12±43.28 µg/g when glucose was used as carbon source at C:N of 40:1

Table 4.4: Effect of carbon sources on the maximum dried cell,total carotenoidsconcentration(X_m ,P_m)and product yield $Y_{p/x}$

Index	X_m**(g/L)**	P_m **(mg/L)**	$Y_{p/x}$ **(µg/g)**
Glucose	35.41±1.28	31.24±0.78	977.12±43.28
Xylose	34.62±1.23	32.96±0.45	732.89±45.83
Glycerol	34.93±1.39	12.76±0.45	692.73±95.53

The results were expressed as mean ± SDdeviations of triplicate measurements

Until now there is no research about production of carotenoids from *R. toruloides Y27012,* so comparing with another yeasts, carotenoids production by the *R.glutinis*at different glucose concentrations showed thatcarotenoid formation enhanced with the increasing of glucose concentration.*R.glutinis*gave at 20 g/L of initial glucose, the highest carotenoids production yield of 13.8 mg/g (Aksu, 2005).

Varying the carbon sources in the culture medium affected carotenoids production by *R.glutinis*mutant 32, glucose yielded a proportionately higher production of carotenoids comparing with glycerol (9.8 mg/L, 2.9 mg/g) and xylose (23.9 mg/L, 2.2 mg/g) (Bhosale, 2001).

R.glutinis gave a carotenoids yield of 135.2 mg/Land 60.07% lipid when glycerol was used as carbon source in the study of Saenge (2011). It is considered as high yield comparing with the results which we had in this research when glycerol was used as carbon source for *R.toruloidsY27012*with yeast extract as nitrogen source, and C/N ratio of 40:1.

In another study, carotenoids yield of red yeast *Sporobolomyces pararoseus*TISTR5213 was 16.55 mg/L after applying RSM when waste glycerol was used as carbon source and after 5 days of fermentation (Manowattana, 2012).

Buzzini (2007) investigated different strains to produce carotenoids in presence of glucose 40g/L and yeast extract 2 g/L.

Carotenoids content varied from 16.4 to 184 µg/g, among the strains investigated, 122.6 µg/g dry cell was obtained from *Rhodosporidium toruloides* DBVPG 6739 and the carotenoids content was 1311 µg/L. Also in other study conducted by Buzzini (2005) the maximum carotenoids production obtained from *Rhodotorula graminis* DBVPG 7021 was 803 µg/g when glucose was used as carbon source after optimization of trace element concentration by RSM.

Lipidyields and content with different carbon sources are given in Table 4.5. *R. toruloidesis*is known as lipogenic yeast and the resultspresented here confirmits ability to accumulate over 40% of its biomass as lipid. However, lipid accumulation was strongly affected by the nature of the carbon source provided.

It was noticed from this research that there was no clear difference between lipid content among glucose and glycerol sources.

Lipid content was 44.78±5.42% and concentration was 15.23±2.06 g/L when glucose was used as carbon source, but considering the concentration, glycerol gave 6.16±0.40 g/L which was lower comparing with glucose and xylose as seen in Table 4.5.This result is almost similar to study by Mereton (1988).

Lipid contents were found to be 42.9% and42.2% when glucose and xylose were used, but lipid content decreased to 34.4% when pure glycerol was used.

Table 4.5: Lipidyields and contentswith different carbon sources

Index	Lipid, g/L	Lipid content, %
Glucose	15.23±2.06	44.78±5.42
Xylose	13.52±2.41	41.77±7.92
Glycerol	6.16±0.40	44.32±4.59

The results were expressed as mean ± SDdeviations of triplicate measurements

The stoichiometry of glucose metabolism indicates that about1.1 moles of acetyl-CoA are generated from 100 g of glucosecatabolized. Xylose can be either metabolized throughthe phosphoketolase reaction, which is the most efficientpathway yielding around 1.2 moles of acetyl-CoA per 100 g of xylose utilized, or the pentose phosphate pathway, where around 1.0 mole of acetyl-CoA isformed per 100 g of xylose utilized. Therefore, ifall the acetyl-CoA produced is channeled towards lipid synthesis, the maximum theoretical yield of SCO produced perglucose consumed is around 0.32 g/g. This value issomewhat higher concerning the fermentation of xylose, assuming that oleaginous microorganismsutilize exclusively the phosphoketolase pathway for xyloseassimilation.With reference to glycerol, the maximum theoretical yield of SCO is around 0.30 g/g.However,evenunder ideal conditions for SCO production, lipid yield on glucose consumed canrarely be higher than 0.22 g/g. (Aggellis, 2011).

Höfer (1971) presented circumstantial evidence that t *Rhodosporidium toruloides* produced a xylose isomerase in xylose synthesis, but was unable to detect this activity in cell-free extracts of this yeast, however, xylose reductase and xylitol dehydrogenase activities were detected in that research.Wiebe and others (2012) compared the lipid content from *R. toruloides* CBS14 between two carbon source glucose and xylose were used. They have found that glucose gave higher biomass and lipid content (9 g/L, 56%) after 66h, whereas xylose gave (8 g/L, 45%) after 113h.Lipid production by *Rhodosporidium toruloides* Y4 in batch fermentation resulted in accumulating of lipids up to 76% of dry biomass when glucose was used as a substrate (Li, 2006). In fed-batch fermentations containingglucose, peptone and yeast extract, *R. toruloides* was reported to accumulate up to 67.5% of lipids and a biomass of 106 g/L in only 134 h (Li, 2007). The same microorganism produced 79 g/L of lipids, in 140 h, with multiple fed-batch of glucose (Zhao, 2010).

4.4 Effect of C/N Ratio on Carotenoids and Lipid production

Since carotenoids and lipid production share acetyl CoA as common precursor, it can not be excluded that there are certain interactions between both production pathways at certain C/N ratios. Effect of C/N ratio on the maximum dried cell, total carotenoids concentration and product yield is presented in Table 4.6.

As can be seen from Table 4.6, the maximum carotenoidsyield was obtained as 1001±17.87 µg/g at C/N ratio of 20 with low biomass (24.76±1.78 mg/L) compared with C/N of 40 and 60.

Table 4.6 : Effect of C/N ratio on the maximum dried cell,total carotenoidsconcentration(X_m ,P_m)and product yield $Y_{p/x}$

Index	X_m (g/L)	P_m (mg/L)	$Y_{p/x}$ (µg/g)
C/N=40	35.41±1.28	31.24±0.78	977.12±43.28
C/N=20	24.76±1.78	22.66±0.78	1001±17.87
C/N=60	53.41±0.93	24.74±1.63	445.38±40.88

The results were expressed as mean ± SD deviations of triplicate measurements

Park (2005) indicated that the best carotenoidproduction occured at a C/N ratio of approximately 44.5 by *Rhodotorula glutinis*. Wherease C/N of 31.6 gave the highest biomass . Also, the C/N ratio of 44.5 observed in that studyfor maximum carotenoids production differed from theresults of Somashekar and Joseph (2000), they investigated carotenoids production from semi-defined minimal salts media with three different C/N ratios by theyeast*R. gracilis*, and found that a C/N ratio of 10 favoredmaximum carotenoids production. This result might be due to the different types of medium and yeast strainused.

In another research conducted by (Libkind, 2004), maximum carotenoids yield (2.32 mg/L) were obtained from *Rhodotorula mucilaginosa* CRUB 0138 at C/N of 5 in culture medium containing 10 and 40 g/L glucose, respectively. Different C/N ratios did not influence carotenoid pigment production but low C/N ratio enhanced biomass yield (Libkind, 2004).The optimized conditions showed that cell growth required low C/N, whereas highest C/N caused increase in lipid content and carotenoids production by *R.glutinis*, at C/N of170 the lipid content and carotenoids production were (39.74 g/L, 148.77 mg/L, respectively) (Saenge, 2011).

It was observed in the medium at different C/N ratios between 10/1 and 70/1, that C/N ratio had no effect on cellular pigment accumulation by *R.glutinis* mutant 32. However, a low ratio resulted in high volumetric production of carotenoids (33 mg/L, 2.90 mg/g) due to high cell mass yield (Bhosale, 2001).

The results of lipid yield and content at different C/N ratios are given in Table 4.7.

In this study it was noticed that the highest lipid content was given when the C/N ratio was 60 with a yield of 16.98±0.79 g/L and lipid content of 49.83±2.53

Table 4.7 : Lipid yield and lipid content at different C/N ratio

Index	Lipid yield, g/L	Lipid content, %
C/N=40	15.23±2.06	44.78±5.42
C/N=20	12.58±2.93	40.28±2.44
C/N=60	16.98±0.79	49.83±2.53

The results were expressed as mean ± SD deviations of triplicate measurements

There are many studies that considered this factor in lipid production from yeasts Nitrogenfeeding should be taken into consideration, because themolecular ratio of carbon-to-nitrogen (C/N) was important for lipid accumulation. It has mentioned that high C/N causes decreasing of AMP (Adenose monophosphate) produced, thus increasing the level of isocitrate which converts to citrate to Acetyl CO-A (Ratledge, 1982).Usually, a C/N ratio of 70 was sufficient to ensure the lipid production process, and a higher C/N ratio gave higher lipid content. However, lipid productivity could be reduced when nitrogen sources were extremely scarce.

For example, cultivation of *R. toruloidesY4* on hydrolysis Jerusalem artichoketuber with a C/N molar ratio of 23 achieved amoderate cellular lipid content of 40% (Hu, 2007). Yeast *Lipomyces starkeyi* produced biomass with a lipid content of 34% whenit was cultured on sewage sludge-based medium with a C/N molar ratio of 18 (Angerbauer, 2008). Very recently, lipid accumulation in the presence of relatively high amounts of organic nitrogenin the growth medium has been documented for the yeast *Cunninghamella echinulata* (Fakas, 2008). They have indicated that complex organic nitrogen sources not readily assimilable had limited detrimental effects on lipid production. In this respect, it shouldbe careful to consider media C/N molar ratios based on total nitrogen analysis if raw materials were employed for lipid production.

Cellular lipid contents were over 70%of their dry biomass when *R. toruloidesY4* cells were cultivated with the initial carbon-to-nitrogen (C/N) molar ratios higher than570, whereas lipid contents were less than 20% if the initial C/N molar ratios were lower than 100 (Li , 2006). Various $(NH_4)_2SO_4$ concentrations were added to culture to give C/N ratios of 22.3, 14.3,9.9, 7.8 and 6.1.

The results are shown that the culture with a C/N molar ratio of 14.3 gave a slightly higher biomass but a lower lipid yield than those with a C/N ratio of 22.3. Both biomass and lipid decreased as culture C/N molar ratios went down. Lipid contents almost held constant at around 60% even the C/N molar ratio reduced to 6.1(Wu, 2010).

Asthe cultural C/N molar ratios in the medium of *R. toruloidesY4* decreased, both biomass and lipid contents decreased concurrently with an increased residual sugar concentration indicating that excess nitrogensource inhibited slightly substrate assimilation and cell growth.Yet, lipid contents almost held constant at near 57% although the C/N molar ratio ranged from 28.3 to 5.7 (Wu, 2011).

4.5 Effect of Activator Addition on Carotenoids and Lipid Production

Some agents, such as detergent additives, oils, surfactants have been suggested as activators for increasing carotenoids and lipid productivity. In order to enhance the carotenoids capability, glucose containing growth medium was supplemented with activators such as Tween 20 and Tween 80 at 0.1 and 1% (v/v). Moreover, 0.1% cotton seed oil, linseed oil,10 g/L of ethanol and 5 g/L acetic acid were also tested. Effect of additives tested on the maximum dried cell, total carotenoidsconcentration and product yield is given in Table 4.8.

It had been noticed that when all additives was added at 0.1% there was no increase in carotenoids yield, but decrease in carotenoids at these concentrations.

Carotenoids formation were decreased with Tween 20, Tween 80, cotton seed oil, and linseed oil when they were added at low concentration (0.1%) together with high concentrations of glucose. These results were consistent with the results of Aksu (2005).

They have also found that, the additives did not have any effect on carotenoids production at high glucose concentrations. When Tween 80 and Tween 20 was added at high concentrations such as 1%(v/v) and glucose was adjusted to have C/N ratio of 20, it was noticed that the carotenoids production and carotenoids concentration increased with a decrease in cell biomass.

Effect of Tween 20, Tween 80, ethanol and acetic acid on the maximum dried cell, total carotenoids concentration and product yield is given in Table 4.9.

Table 4.8 : Effect of additives on the maximum dried cell,total carotenoidsconcentration(X_m ,P_m)and product yield ($Y_{p/x}$)

Index	X_m(g/L)	P_m(mg/L)	$Y_{p/x}$(µg/g)
Control	24.76±1.78	22.66±0.78	1001.51±17.87
0.1 %Tween 20	35.41±1.30	22.40±0.90	606.50±30.16
0.1% Tween 80	28.60±2.80	19.27±0.90	754.68±33.65
0.1% Cotton seed oil	24.05±1.27	ND	ND
0.1% Linseed oil	27.05±0.73	ND	ND
0.1%Tween 80 +0.1% linseedoil	34.72±1.94	20.83±1.97	563.69±29.08
0.1%Tween80 +0.1%cotton seed oil	41.97±1.02	23.18±2.26	682.06±31.99

The results were expressed as mean ± SD deviations of triplicate measurements , ND not detected

As can be seen from Table 4.9, it can be concluded that, at lower glucose concentrations Tween 20 and Tween 80 can increase the carotenoids concentration with little increase in carotenoids yield comparing with control sample. Ethanol gave a high carotenoid yield comparing with control sample and other samples (1732.17±39.45 µg/g) with decrease in biomass.

Cotton seed oil and linseed oil did not have effect on carotenoid production at low concentrations. These oils were added in the presence of of Tween 80 to be sure if the yeast will use these oils. As a result it was found that, the carotenoid yield and carotenoid concentration were the same without cottonseed oil and linseed oil, therefore these oil might have no effect in the activation of the enzymes involved in carotenoids biothensysis when used at at low concentrations with high concentrations of glucose.

Aksu and Eren (2005) reported that the supplementation ofcotton seed oil in the culture medium for growth of *R. mucilaginosa*resulted in an increased production of totalcarotenoids at 5 g/L glucose concentration, the yeast produced 57.6 mg/L carotenoids withcotton seed oil, while 39.5 mg/L carotenoids was produced without the activators.

In thesame study in the presence of 15 g/L glucose medium without additives, carotenoids yield was 13.7 mg/g which was more than when cotton oil was added (9.4 mg/g).

Saenge (2011) had investigated number of surfactants including Tween 80, Tween 20 and gum Arabic for enhancement of lipid and carotenoids production by *R. glutinis* TISTR 5159 yeast. It was cultivated with ammonium sulfate as nitrogen source. There was a significant increase in the amounts of biomass and lipid content and carotenoids production which were 7.07 g/L, 38.15g/L, and 125.94 mg/L, respectively when Tween 20 was added.

Although acetic acid gave a good result when it was added at 5 g/L to a fed-batch culture of *P. rhodozyma* causing increase in carotenoid concentration to 43.87 mg/L comparing with control sample (Kim, 2003). In our carotenoids were not detected may be due to the yeast or to the medium conditions (Table 4.9).

In this study, ethanol increased the carotenoid yield with no change in carotenoid concentration comparing with control sample (1732.17±39.45µg/g, 22.92±0.95 mg/L). Detailed studies revealed that ethanol activates oxidative metabolism with induction of HMG-CoA reductase, which in turn enhances carotenoids production.Similarly, Gu(1997) reported increased carotenoids production (from 1.65 $mg.g^{-1}$ cells to 2.65 $mg.g^{-1}$cells) upon addition of 0.2% (v/v) ethanol to cultures of the yeast *X. dendrorhus*. Also Kim(2003) reported that cell mass and astaxanthin production is stimulated by the addition of ethanol (10 g/L) to a fed-batch culture of *P. Rhodozyma* that resulted with 45.62 mg /L carotenoids.

Table 4.9 : Effect of Tween 20, Tween 80 at 1% (v/v), ethanol 10g/L and acetic acid 5g/L on the maximum dried cell, total carotenoids concentration (X_m ,P_m)and product yield $Y_{p/x}$

Index	X_m**(g/L)**	P_m**(mg/L)**	$Y_{p/x}$**(µg/g)**
Control	24.76±1.78	22.66±0.78	1001.51±17.87
1 % Tween 20	25±1.13	25.26±1.19	1002.79±46.87
1%Tween 80	24±0.46	25±2.07	1014.46±68.44
10 g/L Ethanol	13.33±0.05	22.92±0.95	1732.17±39.45
5 g/L Acetic acid	41.97±1.02	ND	ND

The results were expressed as mean ± SD deviations of triplicate measurements, ND not detected

Polyoxyethylene sorbitan monooleate, generally known as Tween 80, is a non-ionic surfactant and contains amono-unsaturated fatty acid. Tween 80 has been extensively employed as a vehicle for the addition of water insoluble compounds. Tween 80 is known to interfere with the permeability of cell membranes and enhances the nutritional input from the surroundings to the cell body. In addition to Tween 80, there are other Tween series, such as Tween 20, Tween 40 and Tween 60 which contain monolaulate, monopalmitate, monostealateand monooleate, respectively, and these Tween series are aqueous (Scardovi, 1981).

It has been noticed that adding Tween 80, Tween 20 at 0.1% and 1% (v/v) causes increasing in lipid content as can be seen in Table 4.10 and 4.11.

Lipid contents reached to 44.92 %±5.438 and 41.34 %±7.94 , respectively with Tween 80 and 20 at 1% (v/v) compared with the control sample.

The total lipids extracted from *Thraustochytrium* was significantly enhanced by the addition of Tween 80 at (0.1%, 1%) to the culture medium,the total lipids extracted from *T. aureum*ATCC 34304 cells at 96 h were 270 mg/g in thecontrol and 311 mg/g in 1.0% Tween 80, and a significant difference was observed.

Tween80 and Tween 20 may play a role not only as a carbon source but also acts as an enhancer of nutrient uptake into the cell bodies, meaning that the permeability of thecell membrane increases.

It is known that Tween 80 interferes with the cell membrane permeability invarious microorganisms (Scardovi, 1981).Perhaps, the Tween 80 and Tween 20 may enhance to uptake of the essential components including vitamins from surroundings, but there has been no information in thraustochytrids.

Furthermore, the relationship between Tween 20, Tween 80 supplementation and the membrane permeability in thraustochytrids should be investigated. In this study when cotton seed oil and linseed oils were used at 0.1%, it was noticed that *Rhodosporidium toruloidesY27012* did not use these oil and oil appeared on the surface of the flask after the fermentation period.

Ethanol has been considered as a potential substrate for the de novo lipid biosynthesis of the oleaginous microorganisms,in this study lipid content was occured when ethanol at 10 g/L with glucose at 5.21 g/L was used.The results are given in Table 4.11. Compared with the control sample, high amount of lipid content such as 45.21%±0.87 was obtained. It can be considered as a very proper additive since no residual carbonarises from its uses in fermentation processes.

Table 4.10: Lipid yield and lipid contentwith different additives

Index	Lipid (g/L)	Lipid content (%)
Control	12.58±2.93	40.28±2.44
0.1 %Tween 20	13.92±1.00	42.38±1.36
0.1 %Tween 80	12.60±1.05	41.96±3.01
0.1% Cotton seed oil	ND	ND
0.1% Linseed oil	ND	ND
0.1%Tween 80+0.1% linseed oil	15.98±0.77	40.13±3.40
0.1%Tween 80+0.1%cotton seed oil	13.88±0.65	40.99±2.72

The results were expressed as mean ± SDdeviations of triplicate measurements, ND not detected

Lipomyces starkeyi was grown on ethanol maintainedat its optimal concentration of 2.5 g/L. This technique allowed extremely high celldensities to be attained: 153 g/L over 140 h, with a cellular oil content in excess of 50% (Yamauchi, 1983).

The ability to utilize ethanol suggests the presence of an alcohol dehydrogenase, to convert ethanol to acetaldehyde, and an acetaldehyde dehydrogenase to convert acetaldehydeto acetate.

Takinginto consideration that theoretical yieldof 0.54 g of lipid per 1 g of ethanol consumed (Ratledge, 1988).Nevertheless such high conversion yields have never been achieved in the literature with the conversion threshold of ethanol into SCO being around 0.31 g/g.(Ratledge, 1988; Fakas, 2009).

Acetic acid also gave a high lipid content and yield of 61.27%±1.77, 13.07±1.71g/L at 5g/L of acetic acid. Acetic acid has been equally considered as substrates for SCO production. Roux (1995) had used acetic acid as a carbon source and as a result lipid content was 47% of biomass.

The major reasons for this improved productivity would appear to originate from both acetic acid and ethanol feeding directly into the pool of acetyl-CoA that is needed for lipogenesis.

In glucose-grown cells, the main flux of carbon involves glucose uptake, glycolysis, transport of pyruvate into the mitochondrion, conversion of pyruvate into citrate,transport of citrate into the cytosol, and cleavage of citrate by adenosine triphosphate(ATP):citrate lyase to yield acetyl-CoA. This has been well explored in both yeastsand fungi. (Ratledge, 2002).

From the findings of our research, it can be concluded that, acetic acid was better than ethanol for obtaining higher lipid content and concentration even it was used at low concentrations concedring that toxic effects of both ethanol and acetic acids.

Table 4.11: Lipid yield and lipid contentwith different additives

Index	Lipid (g/L)	Lipid content (%)
Control	12.58±2.93	40.28±2.44
1 %Tween 20	10.72±2.20	41.34±7.94
1% Tween 80	8.35±1.23	44.92±5.43
10g/L Ethanol	8.59±1.05	45.21±0.87
5g/L Acetic acid	13.07±1.71	61.27±1.771

The results were expressed as mean ± SDdeviations of triplicate measurements, ND not detected

4.6 Medium Optimization by Response Surface Methodology

Response surface methodology (RSM) was applied to optimize concentration g/L (x_1), yeast extract concentration g/L (x_2) and ethanolconcentration g/L (x_3) for biomass content and production of lipids and carotenoids from *R.toruloids*Y27012 Central composite design (CCD) with 3 center points leading to a total 15 sets of experimentswas carried out as shown in Table 4.12.

From Table 4.13 it is obsorved that the most important effects on biomass incorporation was found as ‘‘ethanol’’.

“Glucose” , “ethanol*ethanol” , and “glucose* ethanol” do not have any effect on biomass as (p>0.05).Wherease “glucose*glucose”, “yeast extract*yeast extract”, “ethanol* ethanol”, and “yeast extract*ethanol” have an effect on lipid content as (p<0.05), and glucose*glucose has a negative effect in lipid content.

For carotenoid yield "yeast extract*yeast extract", "ethanol* ethanol", and "glucose*yeast extract" have an effect on carotenoid yield and the most important effects was ethanol*ethanol in negative way.

Table 4.12: Experimental design matrix and experimental results for the CCD design

	Factors			**Responses**		
	Glucose (g/L)	**Yeast extract (g/L)**	**Ethanol (g/L)**	**Biomass (g/L)**	**Lipid content (%)**	**Carotenoids content (µg/g)**
	x_1	x_2	x_3	Y_1	Y_2	Y_3
1	1(9.79)	1(4.72)	0(10)	13.31±2.09	47.16±1.11	1526.69±132.79
2	1(9.79)	-1(2.72)	0(10)	14.87±1.31	47.48±4.46	1314.72±135.72
3	-1(3.38)	1(4.72)	0(10)	13.10±1.45	41.72±1.98	1471.94±51.13
4	-1(3.38)	-1(2.72)	0(10)	10.44±0.92	45.19±2.45	1538.69±108.83
5	1(9.79)	0(3.72)	1(15)	13.59±1.63	46.17±2.07	1238.08±128.28
6	1(9.79)	0(3.72)	-1(5)	12.77±0.98	43.84±2.70	1444.59±28.26
7	-1(3.38)	0(3.72)	1(15)	10.42±1.9	45.95±0.67	1467.21±21.18
8	-1(3.38)	0(3.72)	-1(5)	11.69±3.09	40.22±1.15	1366.69±56.616
9	0(6.58)	1(4.72)	1(15)	13.09±1.50	65.86±0.95	1276..55±100.11
10	0(6.58)	1(4.72)	-1(5)	13.84±0.88	57.38±0.73	1334.76±79.84
11	0(6.58)	-1(2.72)	1(15)	11.97±1.27	57.18±7.00	1198.80±140.3
12	0(6.58)	-1(2.72)	-1(5)	9.74±1.99	67.91±1.49	1485.76±168.44
13	0(6.58)	0(3.72)	0(10)	10.86±2.37	46.7±0.47	1692.03±210.80
14	0(6.58)	0(3.72)	0(10)	10.64±2.95	48.86±0.47	1636.37±130.75
15	0(6.58)	0(3.72)	0(10)	11.06±2.77	46.92±0.47	1789±80.02

The results were expressed as mean ± SDdeviations of triplicate measurements

The regression equations for biomass (Y_1), lipidcontent (Y_2), and carotenoid production (Y_3) as a function of glucose (x_1), yeast extract (x_2), and ethanol (x_3) are given as followed Eqs:

$$\text{Biomass } (Y_1) = 10.8533 - 0.061x_1 + 0.076x_2 - 1.18x_3 + 0.58{x_1}^2 + 1.06{x_2}^2 + 0.25{x_3}^3 - 0.80x_1x_2 + 0.39x_1x_3 - 0.74x_2x_3 \quad \textbf{(4.1)}$$

$$\text{Lipid content}(Y_2) = 49.4932 - 0.11x_1 - 0.31x_2 - 0.24x_3 - 5.96{x_1}^2 + 7.59{x_2}^2 + 6.99{x_3}^2 + 0.038x_1x_2 - 0.64x_1x_3 + 4.8x_2x \quad \textbf{(4.2)}$$

$$\text{Carotenoids yield } (Y_3) = 1685.21 + 7.64x^1 + 49.41x^2 + 28.04x^3 - 56.25\,x^{12} - 153.567x^{22} - 207.67x^{32} + 175.148x^1x^2 - 58.05x^1x^3 + 57.196x^2x^3 \quad \textbf{(4.3)}$$

Table 4.13: Regression coefficients (β) and significance values (*p*-value) of biomass, lipid content, and carotenoid yieldS

	Biomass(g/L)		**Lipid content(%)**		**Carotenoids yield(µg/g)**	
Variables	**Coefficient (β)**	***P*-value**	**Coefficient (β)**	***P*-value**	**Coefficient (β)**	***P*-value**
Intercept	10.85	<0.0001	47.49	<0.0001	1685.28	<0.0001
x_1	-0.061	0.71	-0.11	0.886	7.64	0.727
x_2	0.076	0.015	-0.31	0.764	49.41	0.131
x_3	-1.18	0.0023	-0.24	0.814	28.04	0.353
x_1^2	0.58	0.0211	-5.96	0.00008	-56.25	0.058
x_2^2	1.06	0.018	7.59	0.0036	-153.567	0.012
x_3^2	0.25	0.455	6.99	0.0052	-207.67	0.0036
x_1x_2	-0.80	0.015	0.038	0.972	175.148	0.05
x_1x_3	0.39	0.136	-0.64	0.575	-58.05	0.104
x_2x_3	-0.74	0.052	4.80	0.0196	57.19	0.20

P-value, level of significance

The graphic plot of predicted values by the model vs. observed experimental values showed a linear distribution for biomass, lipid content, and carotenoids yield (R^2=0.95, 0.96, and 0.92, respectively) for the response, This indicated that up to92 ~ 96 % of the variations in biomass, lipids , and carotenoids can be explained by these equations.

The ANOVA analysis presented in Table 4.14 indicates that the model of biomass was highly appropriate for the prediction since the F_{model} (12.45) value was high compared to the $F_{5,9(table)}$ =3.48 (α=0.05). The model showed no lack of fit (*P*=0.075>0.05).

Table 4.14 ANOVA analyses of the response of biomass

Biomass	DF	SS	MS	F ratio	*P*- value
Total	15	2226.12	148.408		
Constant	1	2193.25	2193.25		
Total corrected	14	32.872	2.348		
Regression	9	31.135	3.459	9.96	0.010
Residual	5	1.736	0.3473		
Lack of fit (model error)	3	1.648	0.549	12.45	0.075
Pure error (replicate error)	2	0.020	0.0441		

DF, Degree of freedom; SS, Sum of squares; MS, Mean square

Also in ANOVA analysis of lipid content and carotenoids yield as in Table (4.2; 4.3) it is noticed that F the model was highly appropriate $F_{model}$for lipid and carotenoids (13.12, 6.50, respectively) were higher than $F_{5,9(table)} = 3.48$. The model of lipid content showed that there is no lack of fit since (P=0.103>0.05).

Also carotenoids yield model showed no lack of fit (P=0.686>0.05) table 4.16

Table 4.15 ANOVA analyses of the response of lipid content

Lipid content	DF	SS	MS	F ratio	*P*- value
Total	15	38056.1	2537.07		
Constant	1	3701.3	3706.13		
Total corrected	14	994.829	71.059		
Regression	9	954.425	106.047	13.123	0.006
Residual	5	40.404	8.080		
Lack of fit (model error)	3	37.578	12.526	8.865	0.103
Pure error (replicate error)	2	2.825	1.413		

DF, Degree of freedom; SS, Sum of squares; MS, Mean square.

Table 4.16 ANOVA analyses of the response of Carotenoids yield

Carotenoids yield	DF	SS	MS	F ratio	*P*- value
Total	15	3.14E007	3.14E006		
Constant	1	3.11E007	3.11E007		
Total corrected	14	382112	27293.7		
Regression	9	352062	39118	6.508	0.026
Residual	5	30050.8	6010.15		
Lack of fit (model error)	3	13872.5	464.15	0.571	0.686
Pure error (replicate error)	2	16178.3	8089.16		

DF, Degree of freedom;SS, Sum of squares; MS, Mean square

Regression models were employed to develop response surface plots as shown in Fig (4.1; 4.2; 4.3).

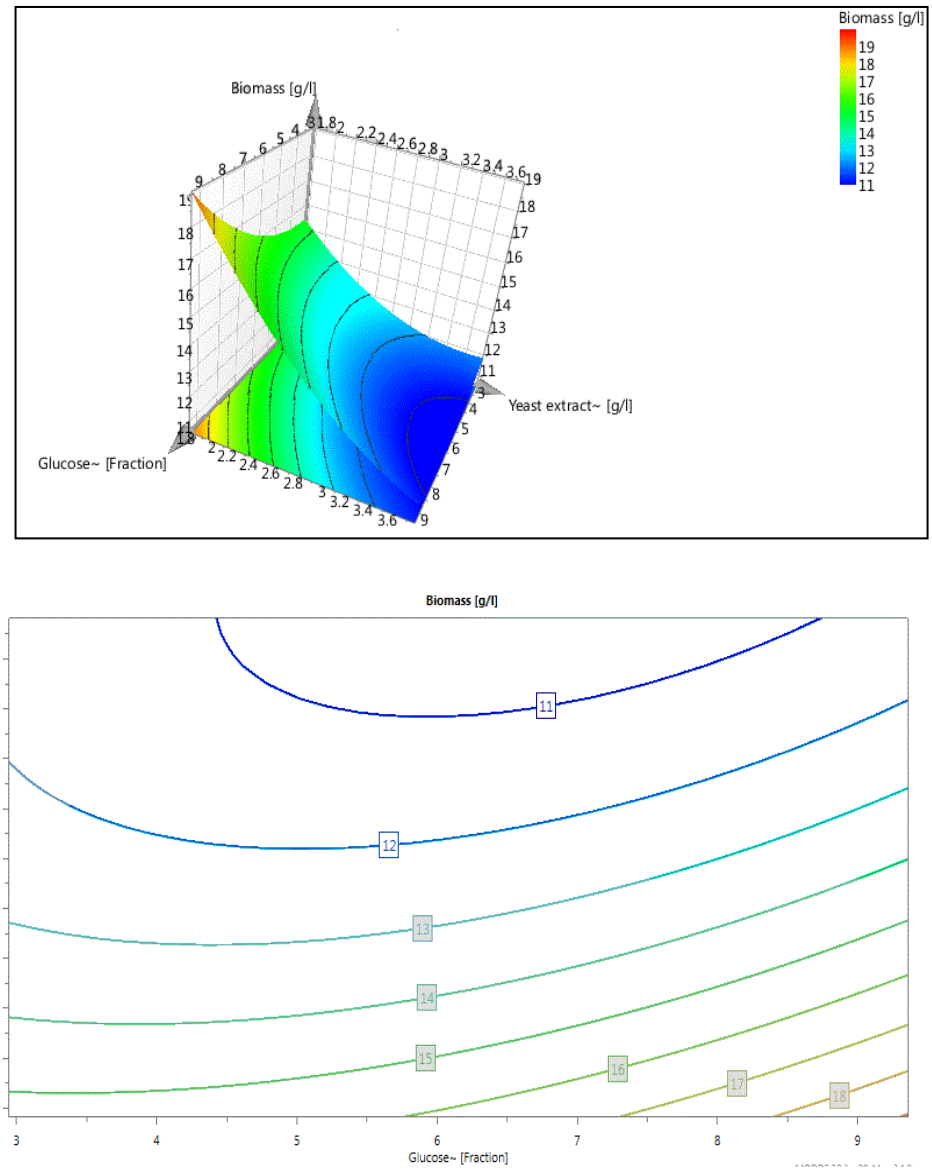


Figure 4.1: Response surface plots and contour plots for the effect of glucose (x_1) and yeast extract (x_2) on biomass

The response surface and contour plots of biomass, lipid content, and carotenoid production illustrated the effects of the Glucose and yeast extract concentrations. Based on response surface plots, the interaction between the two variables and their optimum levels can be easily understood and located.

Fig.(4.1) shows that the biomass increased with increasing glucose concentration and decreasing yeast extract concentraion. Conversely, the maximum biomass (18.78 g/L) was obtained at glucose of 9.28 g/L and decreased yeast extract at 1.78 g/L .

Also from Fig (4.2) it was noticed that maximum lipid content (75.14%) was obtained at a moderate glucose concentration of (6 g/L) and decreased yeast extract cocentration (1.77g/L). It can be noticed that yeast extract at (1.77g/L) concentration gave the maximum biomass and lipid content.

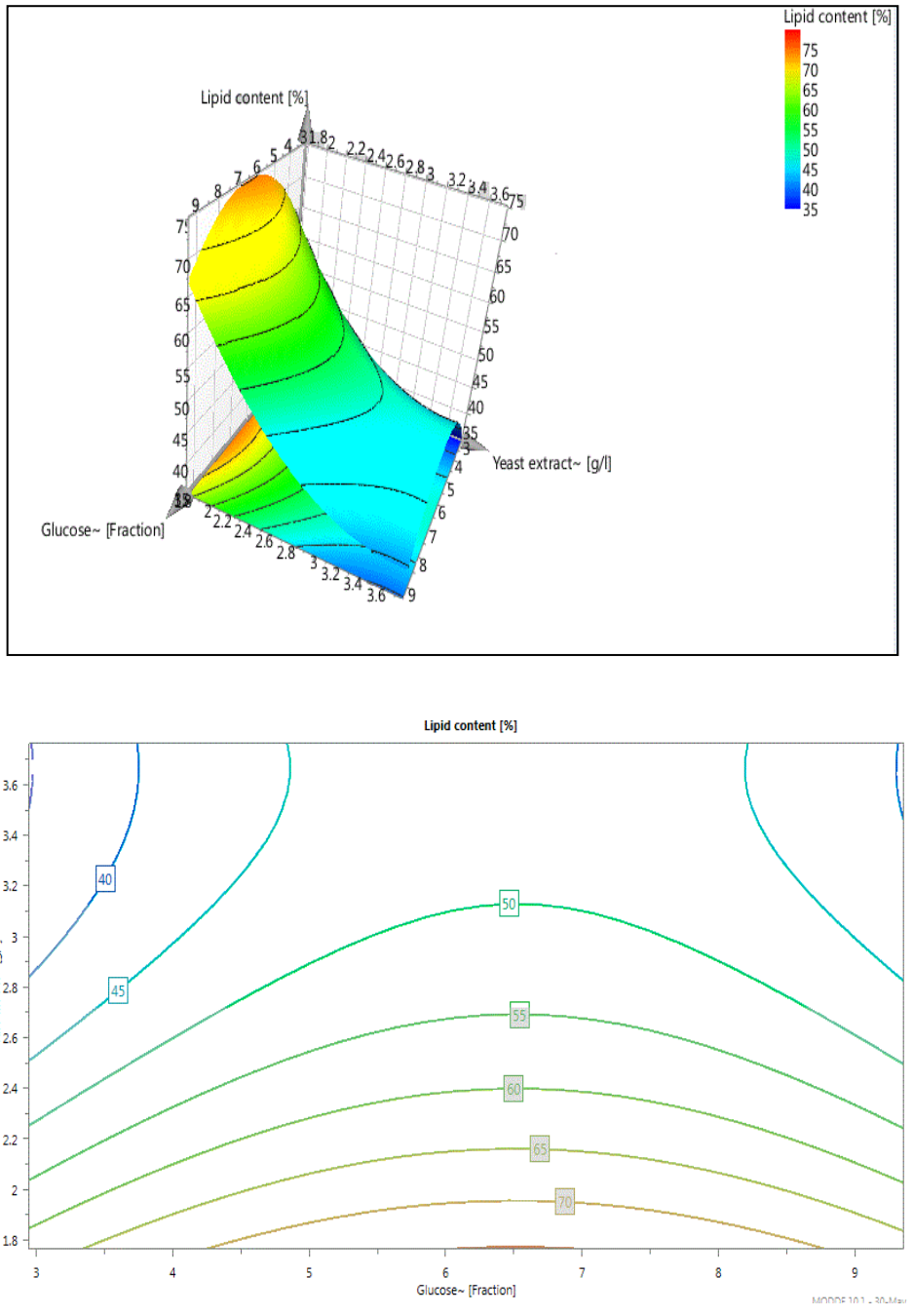
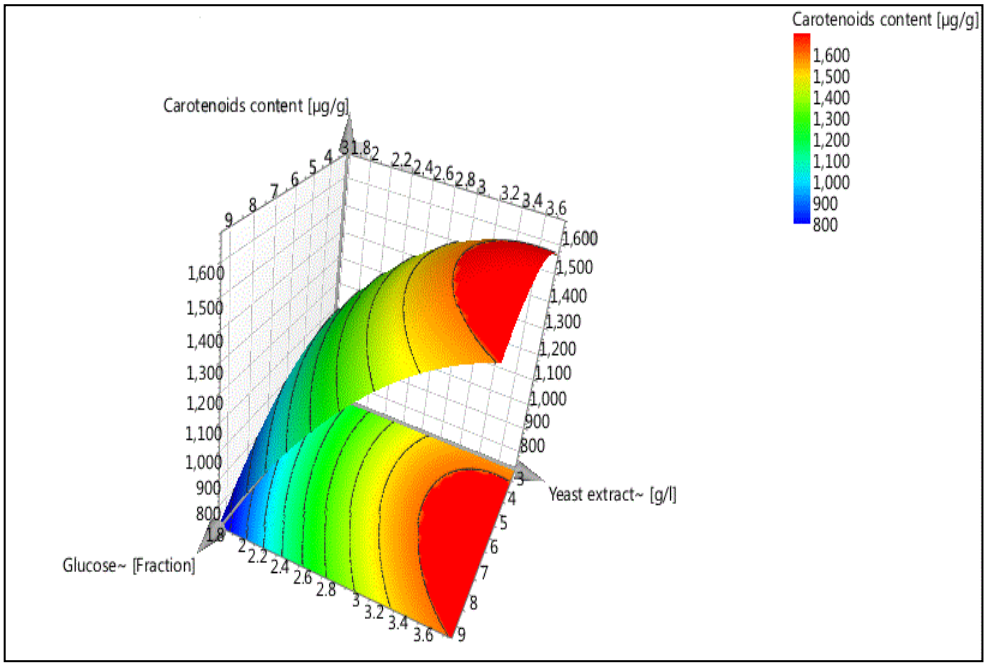


Figure 4.2: Response surface plots and contour plots for the effect of glucose (x_1) and yeast extract (x_2) on lipids content

The maximum carotenoids yield (1680.25 µg/g) was obtained at glucose concentration of 6.43 g/L and relatively high yeast extract concentration of (3.77 g/L) (Fig 4.3).



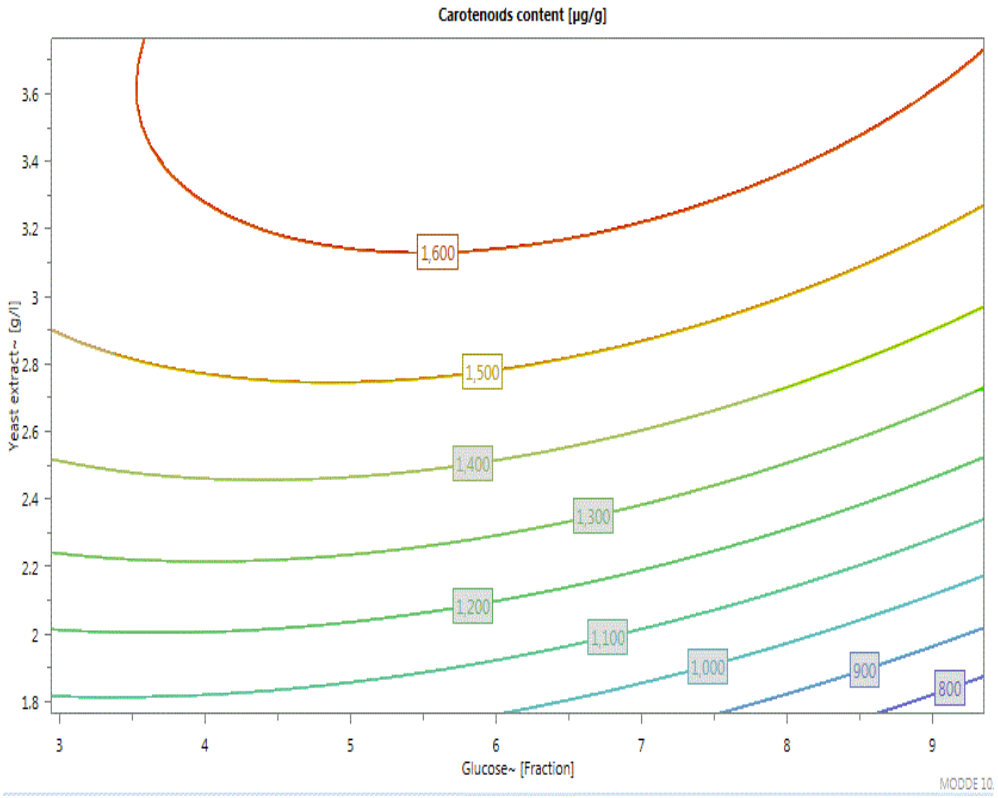


Figure 4.3 : Response surface plotsand contour plots for the effect of glucose (x_1) and yeast extract (x_2) on carotenoid yield

4.7 Fatty Acids Analysis

Lipid sample were transmethylated and analyzed by gas chromatography.

The fattyacid composition showed that four major constituent fattyacids were noticed oleic acid (18:1), palmitic acid (16:0), stearicacid (18:1) and linoleic acid (18:2).

Oliec acid was noticed at 38.54% followed by palmitic acid (20.82%) then palmitoleic acid (12.71%), linoleic acid (12.01%), steatric acid(4.4%) palmitoleic acid. The rest fatty acid were at low ratios.

The major fatty acid compositional profile from *Rhodosporidium toruloidesY27012* was quite similar to that of vegetable oil such as soybean oil,indicating that microbial lipid is of great potential as an alternativefeedstock for the biodieselindustry.

The fattyacid composition of *Rhodosporidium toruloides* in Zhao (2011) study showed that four major constituent fatty acids were oleic acid (18:1), palmitic acid (16:0), stearicacid (18:0) and linoleic acid (18:2). The sum of palmiticacid and oleic acid contents was over 80%. Also Wu (2011) noticed when *Rhodosporidium toruloides*Y4 cultivated using amedium with an initial carbon-to-sulfur (C/S) molar ratio of 46,750, that 90% of the fatty acid composed of oleic acid (18:1) followed by palmitic acid (16:0) and stearic acid (18:0). In other research to Wu, It was noticed that Oleic acid (18:1) was the most abundant one(60%), followed by palmitic acid (16:0) and stearicacid (18:0), and these three fatty acids accounted for over 90% ofthe total fatty acids when *Rhodosporidium toruloides* Y4 was cultivated at C/P ration of 9552.(Wu, 2010)

After the fatty acids in the extracted lipids from*R. Toruloides 21167* grown in the hydrolysate of cassava starch in the 2-l fermentor were transmethylated and analysed by gas chromatography, the results indicated that over 96.8% of the fatty acids from the yeast strain 21167 was C16:0,C18:0,C18:1and C18:2, especially C18:1(53.34%) while C14:1and C16:1 fatty acids were only 1.7% and 1.5%.(Wang, 2012).

5 CONCLUSIONS

Carotenoids are important molecules that improve foodsquality due to their high nutritional value. For these reasons, carotenoid application as colorants has increasedthe interest of industries and scientists to develop lowcost process for carotenoid production. Biotechnologicalproduction mainly provides economics advantages oversynthetic or extracted plant carotenoids.

In this study, carotenoids extraction was done by two methods ultrasonic (US) assisted and HCl assisted extractrion techniques comparing with control technique (grinding) from *Rhodosporidium toruloides* Y27012. HCl-assisted extraction of carotenoids was found to be the most effective method with a carotenoid yield of 977±43.25 µg/g and concentration of 31.24±0.78 mg/L, compared with US method and control.

Among the nitrogen sources that were examined, yeast extract was found to be the best nitrogen source for carotenoids yield and lipid content (977.12 ± 43.25 µg/g and 41.71±1.02 %,) followed by peptone and ammonium sulfate.

Assimilation of different sugars as carbon source by the red yeast *Rhodosporidium toruloidesY27012*for its growth and pigmentation showedthat highest carotenoids yield was 977.12±43.28 µg/g when glucose was used as carbon source at C/N ratio of 40, whereas It was noticed from this research that no clear difference between lipid content was found when glucose and glycerol sources were used.

The effect of C/N ratio was examined to study its effect on lipid and carotenoids production. The maximum carotenoidsyieldwas obtained at C/N ratio of 20 (1001±17.87 µg/g) compared with low medium nitrogen contents at C/N ratio of 40 and 60. But lipid content increased with the increase in C/N (low nitrogen), and the highest lipid content was noticed at high C/N ratio 60 (49.83±2.53%, 16.98±0.79 g/L).

When some additives were added at low concentration such as 0.1% (v/v), they did not have any effect on carotenoid yield, but carotenoid concentration was still almost the same around 20 mg/L.

But when Tween 80 and Tween 20 were added at high concentration such as 1% (v/v) and glucose was adjusted to have C/N ratio of 20, an increase in Lipids content, carotenoid yield and carotenoid concentration were

noticed. Ethanol addition (10 g/L) increased the carotenoid yield with no change in carotenoid concentration compared with control sample (1732.17±39.45µg/g, 22.92±0.95 mg/L), lipid content increased until 44.92±5.43% from 40.28±2.44% in control sample

Because of the importance of RSM in reducing process variability, development time, and overall costs,Respone surface methodology (RSM) was applied for optimizing the medium additives for the production of biomass, carotenoids and lipids. The graphic plot of predicted values by the model vs. observed experimental values showed a linear distribution for biomass , lipid content, and carotenoid yield(R^2=0.947, 0.959, and 0.921, respectively) for the response, This indicated that up to 92 ~ 96 % of the variations in biomass, lipids , and carotenoids can be explained by these equations.

ANOVA anlysis indicate that the model of biomass, lipids content, carotenoids yield were highly appropriate for the prediction since the F_{model} values was high compared to the F_{table}. It is obsorved that the most important effects on biomass incorporation was found as ethanol. "Glucose" , "ethanol*ethanol" , and "glucose* ethanol" do not have any effect on biomass as ($p>0.05$). Wherease "glucose*glucose", "yeast extract*yeast extract", "ethanol* ethanol", and "yeast extract*ethanol" have an effect on lipid content as ($p<0.05$).

While "yeast extract*yeast extract", "ethanol* ethanol", and "glucose*yeast extract" have effects on carotenoid yield . It was noticed after optimization by RSM that the maximum biomass (18.78 g/L) was obtained at glucose of 9.2819 g/L and decreased yeast extract at 1.78 g/L , alsomaximum lipid content (75.1405%) at decreased yeast extract ratio (1.77g/L).The maximum carotenoids yield (1680.25 µg/g) was obtained at glucose concentration of 6.43 g/L and relatively high yeast extract concentration of (3.77 g/L)

The fatty acid composition showed that four major constituent fatty acids were noticed oleic acid (18:1), palmitic acid (16:0), stearic acid (18:1) and linoleic acid (18:2). Oliec acid was noticed at 38.54% followed by palmitic acid (20.82%) then palmitoleic acid (12.71%), linoleic acid (12.01%), steatric acid(4.4%) palmitoleic acid. The rest fatty acid were at low ratios.Untill now there is no reserches for optimization carotenoids production from *Rhodosporidium toruloidesY27012* , so further studies are needed to explain effect of the growth conditions like temperature, light, PH, and areation for carotenoids production from this microrganisim.On the otherhand low-cost substrates as agro-industrialwastes should be tried in the medium growth as an alternetive of carbon and nitrogen souces.

Also in further study, metabolic engineering strategies may be can be done between *Rhodosporidium toruloidesY27012* and noncarotenoginous microrganisms

REFERENCES

Aggelis,G. and J.Sourdis. (1997). Prediction of lipid accumulation-degradation in oleaginous micro-organisms growing on vegetable oils. *Antonie Van Leeuwenhoek* 72:159-165.

Aggelis G ,Seraphim Papanikolaou S (2011). Lipids of oleaginous yeasts. Part I:*Biochemistry of single cell oil production, Eur. J. Lipid Sci.T echnol* 1031–105 .

Aksu, Z.; Eren, A.T.(2005). Carotenoids production by the yeast *Rhodotorula mucilaginosa*, Use of agricultural wastes as a carbon source. *Process Biochemistry*.40, 2985–2991.

An GH (2001). Improved growth of the red yeast, *Phaffia rhodozyma(Xanthophyllomyces dendrorhous)*, in the presence of tricarboxylic acid cycle intermediates. *Biotechnol Lett* 23:1005–1009

Andrews, B.A.; Asenjo, J.A.(1987). Enzymatic lysis and disruption of microbil cells. *Trends in Biotechnology*. 5: 273–2.

Angerbauer C ., M. Siebenhofer, M. Mittelbach and G.M.Guebitz. 2008 . C on version of sewage sludge into lipids by *Lipomyces starkeyi* for biodiesel production. Bioresourse. *Technol*. 99:3051-3056.

Anon. (1997). A summary of the Application for the Evaluation of Lyc-O-Mato lycopene oleoresin from tomatoes under *European Regulation for extension of food use(EC)* No. 258/97.

AOAC international.(2000). Official Methods of Analysis of AOAC international, 16th ed., Arlington, VA, 45.1.03.

Aksu, Z.; Eren, A.T.(2005) Carotenoids production by the yeast *Rhodotorula mucilaginosa*, Use of agricultural wastes as a carbon source. *Process Biochemistry*, 40, 2985–2991.

Armstrong GA. (1994). Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants. *J Bacteriol* 176:4795–4802

Armstrong GA. (1997). Genetics of eubacterial carotenoid biosynthesis: a colorful tale. *Annu Rev Microbiol* 51:629–659.

Ausich RL, Brinkhaus FL Mukharji I, Proffitt J, Yarger J, Yen HB. (1997). β-carotene biosynthesis in genetically engineered hosts US patent 5,656,472.

Bhosale P, Gadre RV. (2001). Production of β -carotene by a *Rhodotorula glutinis* mutant in sea water medium. *Bioresour Technol* 76:53–55

Bhosale P, Gadre RV. (2001). Production of β-carotene by a mutant of *Rhodotorula glutinis*. *Appl Microbiol Biotechnol* 55:423–427

Bhosale P., Gadre RV. (2002). Manipulation of temperature and illumination conditions for enhanced -carotene production by mutant 32 of *Rhodotorula glutinis*. *Lett Appl Microbiol* 34:349– 353.

Bhosale P., Bernstein PS. (2005). Microbial xanthophylls. *Appl Microbiol Biotechnol* 68:445–455.

Botham, P.A and C. Ratedge. (1979). A biochemicial explanation for lipid accumulation in *Candida* 107 and other oleaginous microorganisms. J. Gen. *Microbial*. 114:361-375.

Borowitzka LJ. (1992). Beta-Carotene production using algal biotechnology. *J Nutr Sci Vitaminol* (Tokyo) 38:248–250.

Boussiba S., Fan L., Vonshak A. (1992). Enhancement and determi-nation of astaxanthin accumulation in green alga *Haematococcus pluvialis*. *In: Packer L (ed) Methods Enzy*mol 214:386–391.

Britton G. In., GAF Hendry, JD Houghton, eds.(1992). Natural Food Colorants. Glasgow, UK: *Blackie*, p 141.

P, Martin A (1999) Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin. *Bioresour Technol* 71:41–44.

Buzzini, P.; Martini, A. (2000). Production of carotenoids by strains of *Rhodotorula glutinis* cultured in raw materials of agro-industrial origin. *Bioresource Technology*.71: 41–44.

Buzzini, P.; Martini, A.; Gaetani, M.; Turchetti, B.; Pagnoni, U.M.; Davoli, P. (2005) Optimization of carotenoid production by*Rhodotorula graminis* DBVPG 7021 as a function of trace element concentration by means of response surface analysis. *Enzyme and Microbial Technology*, 36, 687–692.

Buzzini P, Innocenti M, Turchetti B, Libkind D, van Broock M, Mulinacci N. (2007). Carotenoid profiles of yeasts belonging to the genera *Rhodotorula, Rhodosporidium, Sporobolomyces* and *Sporidiobolus*. Can. *J. Microbiol*. 53: 1024-1031.

Chen F. (2005). Isolation and puri fi cation of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography. *J Chromatogr A* 1064:183–186.

Ciegler A. (1965). Microbial carotenogenesis. In: Wayne WU (ed) Advances in applied microbiology. *Academic, New York*, pp 1–34.

Costa, I.,Martelli, H.L., Da Silva, I.M. & Pomeroy, D. (1987). Production of β-carotene by a *Rhodotorula* strain. Biotechnology Letters 373.

Davies, B. H.(1965). "Analysis of Carotenoid Pigments," In: T. W. *Goodwin, Ed., Chemistry and Biochemistry of Plant Pigments, Academic Press*, New York, 1965, pp. 489- 532.

Davoli, P.; Weber, R.W.S. (2002). Carotenoid pigments from red mirror yeast *Sporobolomyces roseus*.*Mycologist*16:103–110.

Davoli, P.; Mierau, V.; Weber, R.W.S.(2004). Carotenoids and fatty acids in red yeasts *Sporobolomyces roseus* and *Rhodotorula glutinis*. *Applied Biochemistry and Microbiology*. 40, 392–397.

De la Fuente JL. (2010). High-titer pro-duction of astaxanthin by the semi-industrial fermentation of *Xanthophyllomyces dendrorhous* . *J Biotechnol* 148:144–146.

Del Campo JA., Moreno J., Rodriguez H., Anggeles Vargas M., Rivas J., Guerrero MG. (1999). Carotenoid content of chlorophycean microalgae: factors determining lutein accumulation in *Muriellopsis sp*. (Chlorophyta). *J Biotechnol*76:51–59.

Del Campo JA. (2001). Lutein production by *Muriellopsis sp*. in an outdoor tubular photobioreactor. *J Biotechnol* 85:289–295.

Deming, C., Yonbin, H., & Zhenxin, G. (2006). Application of statistical methodology to the optimization of fermentative medium for carotenoids production by *Rhodobacter sphaeroides*. *Process Biochemistry*, 41, 1773–1778.

Desai HG, Modi VV. (1977). Stimulation of carotenogenesis by penicillin inBlakeslea trispora.*Phytochemistry* 16:1373–1376.

Di Mascio, P.; Murphy, M.E.; Sies, H. (1991). Antioxidant defense systems, the role of carotenoids, tocopherols and thiols. *American Journal of Clinical Nutrition.*53:194S–200S

Ducrey Sanpietro LM, Kula MR. (1998). Studies of astaxanthin biosynthesis in *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*). *Effect of inhibitors and low temperature. Yeast* 14:1007–1016.

Economou CN, Vasiliadou IA, Aggelis G, Pavlou S, Vayenas DV. (2011) Modeling of Oleaginous Fungal Biofilm Developed on Semi-Solid Media. *Bioresource Technol* 102: 9697-9704.

Evan ,CT and C Ratledage (1983). Biochemical activities during lipid accumulation in *Candida curvata* . *Lipid*. 18:630-635.

Fakas, S., Bellou, S., Makri, A., Aggelis, G. (2009). Single cell oil and gamma-linolenic acid production by *Thamnidium elegans*grown on raw glycerol, in: Aggelis, G. (Ed.),*MicrobialConversions of Raw Glycerol, Nova Science Publishers Inc.*,*New York* (USA) pp. 85–99.

Fang, T.J.; Wang, J.M. (2002). Extractability of Astaxanthin in a mixed culture of a carotenoid over-producing mutant of *Xanthophyllomyces dendrorhousand* Bacillus circulansin two stage batch fermentation. *Process Biochemistry*. 27: 1235–1245.

Feofilova, E.P.; Tereshina, V.M.; Memorskaya, A.S. (1995). Regulation of lycopene biosyntheseis in mucurous fungus Blakeslea trisporaby pyridine derivatives. *Mikrobiologiya*, 64,734–740.

Ferrao, M.; Garg, S.(2012). Shake flask optimization of β-carotene production in *Rhodotorula graminis* RC04. *African Journal of Biotechnology*, v. 11, n. 52, p. 11431-11437.

Flores-Cotera LB, Martin R, Sanchez S. (2001). Citrate, a possible precursor of astaxanthin in Phaffia rhodozyma: in Xuence of varying levels of a ammonium, phosphate and citrate in a chem-ically deWned medium. *Appl Microbiol Biotechnol* 55:341–347.

Folch, J., Lees, M., Stanley, G.H.S. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem*. 226, 497–509.

Francis GW. (1975). Variations in the carotenoid content of *Chlamydomonas reinhardii* throughout the cell cycle. Arch Microbiol. 104:249–254.

Francisco,D., and Ocatavia, P. (1997). Effects of enzymatic treatment on carotenoids extraction from marigold flowers(*Tagetes erecta*).*Food Chemistry*, 58(3), 255-258.

Frengova, G.; Simova, E.D.; Pavlova, K.; Beshkova, D.M.; Grigirova, D. (1994) Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafilterate. *Biotechnology and Bioengineering*.44, 888–894.

Fukuri, S and Tanaka, A.(1979).Peroxisomes of alkane and methanol grown yeasts. Journal of Applied Biochemstry 1: 171-201.

Giovannucci E, Clinton SK. (1998). Tomatoes, lycopene, and prostate cancer. *Proc Soc Exp Biol Med* 218:129–139.

Goodwin TW .(1980). Biosynthesis of carotenoids. In: Goodwin T(ed) The biochemistry of the carotenoids, vol I. *Chapman and Hall, Londo*n, pp 33–7

Goodwin.T, Britton.G. (1988). Distribution and Analysis of Carotenoids. In:*Plant Pigments, Aca-demic Press, London, UK.*

Goodwin TW. (1993). Biosynthesis of carotenoids: an overview. In:Packer L (ed) Carotenoids. Part B: metabolism, genetics and biosynthesis. *Methods Enzymol* 214:330–340.

Gu WL, An GH, Johnson EA. (1997). Ethanol increases carotenoid production in *Phaffia rhodozyma. J Ind Microbiol Biotechnol* 19:114–117.

Gu, Z.; Chen, D.; Han, Y.; Chen, Z., Gu F. (2007). Optimization of carotenoids extraction from *Rhodobacter sphaeroides*. *LWT*,41, 1082–1088.

Grill, C.O., MJ.Hall and C.Ratledge. (1997). Lipid accumulation in an oleaginous yeast (*Candida* 107) growing on glucose in single stage continuous culture. *Appl. Environ. Microbiol*. 33:231-239.

Haard, N.F. (1988). Astaxanthin formation by yeast *Phaffia rhodozymaon* molasses.*Biotechnolog Letters*, 10, 609–614.

Hak AE, Ma J, Powell CB, Campos H, Gaziano JM, Willett WC, Stampfer MJ. (2004). Prospective study of plasma carotenoids and tocopherols in relation to risk of ischemic stroke. *Stroke* 35:1584–1588.

Hayman EP, Yokoyama H, Chichester CO, Simpson KL. (1974). Carotenoid biosynthesis in *Rhodotorula glutinis*. *J Bacteriol* 120:1339–1343.

Heo,S. K.,Lee, and S.D.Ha. (2009). A predictive model for the growth of rate of *bacillus cereues* in broth by respone surface methodology .*Biotechnol Bioproc* .Eng14:202-206.

Heo SJ. (2010) Evaluation of anti-in fl ammatory effect of fucoxanthin isolated from brown algae in lipopolysaccharide-stim-ulated RAW 264.7 macrophages. *Food Chem Toxicol* 48:2045–2051.

Hofer, M., Brand,K., Deckner(1971). Importance of the pentose phosphate path way for D-glucose catabolisim in the obligatory aerobic yeast *Rhodotorula gracilis*. *Biochemical Journal* 123:855-863.

Hu ZC, Zeng YG, Wang Z, Shen YC. (2006). pH control strategy in astaxanthin fermentation bioprocess by *Xanthophyllomyces dendrorhous*. *Enzyme Microb Technol* 39:586–590.

Hu, C.M., Zhao, X., Zhao, J., Wu, S.G., Zhao, Z.K. (2009). Effects of biomass hydrolysis by-products on oleaginous yeast *Rhodosporidium toruloides*. *Bioresour. Technol*.100, 4843–4847.

Jacobson, GK, Jolly SO, Sedmark JJ, Skatrud TJ, Wasileski JM. (2000). Astaxanthin over-producing strains of *Phaffia rhodozyma*.Meth-od for their cultivation and their use in animal feeds. *United States Patent* 6,413,736.

Jacobson GK, Jolly SO, Sedmark JJ, Skatrud TJ, Wasileski JM. (2000). Astaxanthin over-producing strains of *Phaffia rhodozyma*.Meth-od for their cultivation and their use in animal feeds. *United States Patent* 6,413,736.

Jane E. Holdsworth, Marten Veenhus and Colin Ratledge.(1988). Enzyme activites in oleaginous yeast accumulating and utilizing Exogenous or Endogenous lipid. *Journal of General Microbiology* 134:2907-2915.

Johnson, E.A.; Schroeder, W.A. (1995). Microbial carotenoids. *Advances in Biochemical Engineering and Biotechnology*. 53, 119–178

Jin, ES. (2003). A mutant of the green alga *Dunaliella salina* constitutively accumulates zeaxanthin under all growth conditions. *Biotechnol Bioeng* 81:115–124

Kim SJ, Kim GJ, Park DH, Ryu YW. (2003). High-level production of astaxanthin by fed-batch culture of mutant strain *Phaffia rhodozyma* AJ-6-1.J *Microbiol Biotechnol* 13:175–181

Kim BK, Park PK, Chae HJ, Kim EY. (2004). Effect of phenol on β-carotene content in total carotenoids production in cultivation of *Rhodotorula glutinis*. *Korean J ChemEng* 21:689–692.

Kim SW, Kim JB, Jung WH, Kim JH, Jung JK (2006). Over-production of beta-carotene from metabolically engineered *Escherichia coli*. *Biotechnol Lett* 28:897–904.

Klaui H, Bauerfeind CJ. (1981). Carotenoids as food colors. In: Bauerfeind JC (ed) Carotenoids as colorants. *Academic, New York, pp* 48–292

Latha BV, Jeevaratnam K, Murali HS, Manja KS (2004) Influence of growth factors on carotenoid pigmentation of *Rhodotorula glutinis DER-PDY* from natural source. *Indian J Biotechnol* 4:353–357.85.

Libkind D, Brizzio S, Broock MV. (2004). *Rhodotorula mucilaginosa*, a carotenoids producing yeast strain from a patagonian high-altitude lake. *Folia Microbiol*. 2004;49: 19-25.

Libkind, D. and van Broock, M. (2006). Biomass and Carotenoid Pigment Production by Patagonian Native Yeasts, World J. *Microbiol. Biotechnol*

Li, Y.H., Liu, B., Zhao, Z.B., Bai, F.W.(2006). Optimization of culture conditions for lipid production by *Rhodosporidium toruloides*. *Chin. J. Biotechnol*. 22, 650–656.

Li Y.H, Zhao Z.K, Bai F.W. (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloidesY4* in fed-batch culture. *Enzyme Microb Technol* 41:312–317.

Liu, H. (2009). .Comparative proteomic analysis of *Rhodosporidium toruloides* during lipid accumulation. *Yeast* 26,553–566.

Malisorn, C.; Suntornsuk, W. (2008). Optimization of β-carotene production by *Rhodotorula glutinis DM28* in fermented radish brine. *Bioresource Technology*.99, 2281–2287.

Manowattana , Phisit Seesuriyacha , Charin Techapu and Thanongsak Chaiyaso.(2012). Optimization of Carotenoids Production by Red Yeast *Sporobolomyces pararoseus* TISTR5213 Using Waste Glycerol as the Sole Carbon Source . *KKU Res*. J;17(4):607-621.

Margalith P, Meydav S. (1968). Carotenogenesis in the yeast *Rhodotorula mucilaginosa*. *Phytochemistry* 7:765–768.

Marova I, Carnecka M, Halienova A, Certik M, Dvorakova T, Haronikova A.(2011) Use of several waste substrates for carotenoid-rich yeast biomass production. *Env Manag*,95:338–342.

Martin, A.M.; Lu, C.; Patel, T.R. (1993). Growth parameters for *Rhodotorula rubra* grown in peat extracts. *Journal of Fermentation and Bioengineering*. 76, 321–325.

Mayne ST. (1996). Beta-carotene, carotenoids, and disease prevention in humans. *FASEB* J 10:690–701.

Meesters, P.A.E.P.,G.N.M Huijberts and G.Eggink. (1996). High cell density cultivation of lipid accumulating yeast *Cryptococcus curvatus* using glycerol as carbon source .*Appl.Microbial Biotechnol* 45:575-579.

Mehta BJ, Obraztsova IN, Cerda-Olmedo E. (2003). Mutants and intersexual heterokaryons of *Blakeslea trisporafor* production of beta-carotene and lycopene. *Appl Environ Microbiol* 69:4043–4048.

Meyer, P.S.; Du Perez, J.C. (1994). Astaxanthin production by a *Phaffia rhodozyma* mutant on grape juice. *World Journal of Microbiology and Biotechnolog* 10, 178–183.

Michelon M, Matos de Borba T, Ruan de Silva R, Veiga Burkert CA, de Medeiros Burkert JF. (2012). Extraction of carotenoids from *Phaffia rhodozyma*:A comparison between different techniques of cell disruption. *Food Sci Biotech*.21:1–8.

Misawa N, Nakagawa M, Kobayashi K, Yamano S, Izawa Y, Nakamura K, Harashima K. (1990). Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*. *J Bacte*riol 172:6704–6712.

Misawa.N, H. Shimada (1997). Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts, J. *Biotechnol.* 59 :169–181.

Nasrabadi M, Razavi S.(2011). Optimization ofβ-carotene production by a mutant of the lactose-positive yeastRhodotorula achenoriumfrom whey ultrafiltrate.*Food Sci Biotech*,20:445–454.

Nelis HJ, De Leenheer AP. (1991). Microbial sources of carotenoid pigments used in foods and feeds. *J Appl Microbiol* 70:181–191

Orset SC, Young AJ. (1999). Low-temperature-induced synthesis of α-carotene in the microalga *Dunaliella salina*(Chlorophyta). *J Phycol* 35: 520–527

Papanikolaou S, Aggelis G. (2002). Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stagecontinuous culture. *Bioresour technol* 82:43–49.

Polulyakh OV, Podoprigova OI, Eliseev SA, Ershov YV, Byk-hovsky VY, Dmitrovski AA. (1991). Biosynthesis of torulene and torularhodin in the yeast *Phaffia rhodozyma*. *Prikl Biokhim Mi-krobiol* 27:541–545.

Politino,M., S.M.Tonzi, W.V. Burnett, G, Romancik and Usher J.J (1997). Purification and characterization of cephalosporin esterase from *Rhodosporidium toruloides* .Appl.Environ.Microbial.03(12):4807-4811.

Raja R. (2007). Exploitation of Dunaliella for β-carotene production. *Appl Microbiol Biotechnol* 74:517–523

Raja R, Hemaiswarya S, Rengasamy R. (2007). Exploitation of *Dunaliella* for beta-carotene production. *Appl Microbiol Biotechnol* 74:517–523.

Ratledge, C. 1982 Microbial oils and fats: an assessment of their commercial potential. *Progress in Industrial Microbiology* 16, 119-206.

Ratledge, C. (1988). Biochemistry, stoichiometry, substrates and economics, in: *Moreton, R. S. (Ed.), Single Cell Oil, Longman Scientific & Technical, Harlow (UK)* 1988, pp. 33–70.

Ratledge, C. (2002). Regulation of lipid accumulation in oleaginous microorganisms. *Biochem Soc Trans* 30:1407–1450.

Ratledge, C and J.P. Wynn. (2002). The biochemistry and molecular biology of lipid accumulation in oleaginous microorganisms. *Adv. Appl. Microbial*. 51:1-51.

Ruther A, Misawa N, Boger P, Sandmann G. (1997). Production of zeaxanthin in *Escherichia coli* transformed with different carotenogenic plasmids. *Appl Microbiol Biotechnol* 48:162–167.

Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T. (2011). Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids and carotenoids. *Process Biochem*. 46:210–8.

Saenge C, Cheirsilp B, Suksaroge TT, Bourtoom T. (2011). Efficient Concomitant Production of Lipids and Carotenoids by Oleaginous Red Yeast *Rhodotorula glutini*s Cultured in Palm Oil Mill. *Effluent and Application of Lipids for Biodiesel Production*. 16-23:33.

Sakaki H, Nochide H, Nakanishi T, Miki W, Fujita T, Kome-mushi S. (1999). Effects of culture conditions on the biosynthesis of carotenoids in *Rhodotorula glutinisN21*. *Seibutsu-kogaku Kaishi* 77:55–59.

Sakaki H, Nakanishi T, Tada A, Miki W, Komemushi S. (2001). Activation of torularhodin production by *Rhodotorula glutinis* using weak white light irradiation. *J Biosci Bioeng* 92:294–297.

Sandmann,G. (2001). Carotenoid biosynthesis and biotechnological application, Arch. *Biochem. Biophys*. 385:4–12.

Stauffer,C.E. (1996).Fats and oils. *Eagan Press,St. Paul, Minnesota*.

Shiroishi M, Tada M. (1982). Mechanism of photo regulated carotenogenesis in *Rhodotorula minuta*.I. Photocontrol of carotenoid production. *Plant Cell Physiol* 23:541–547.

Simpson KL, Nakayama TOM, Chichester CO. (1964). Biosynthesis of yeast carotenoids. *J Bacteriol*. 88:1688–169

Simova, E.D.; Frengova, G.I.; Beshkova, D.M. (2003). Effect of aeration on the production of carotenoid pigments by *Rhodotorula rubra-lacobacillus caseisub*spcasei co-cultures in whey ultrafilterate. *Naturforsch*. 58c, 225–229.

Somashekar ,D. and R. Joseph. (2000). Inverse relationship between carotenoids and lipid formation in Rhodotola gracilis according to C/N ratio of growth medium . World J.*Microbial Biotechnol* 16:491-393.

Stabnikova EV, Slyusarenko TP, Burik MA, Polishchuk GI. (1979). Optimization of the nutrient medium for the cultivation of carotene-forming yeast. *Kharchova Promislovist* 25:55–59.

Stahl W, Sies H. (1996). Lycopene-A biologically important carotenoid for humans. *Arch Biochem Biophys* 336: 1–9.

Tada M, Shiroishi M. (1982). Mechanism of photoregulated carotenogenesis in *Rhodotorula minuta*. II. Aspects of photoregulative reaction. *Plant Cell Physiol* 23:549–556

Thorpe, R.F. and C.Ratledge. (1972). Fatty acid distribution in triglycerides of yeasts grown on glucose or n-alknes. *J.Gen Microbial*. 72:151-163.

Toaka,Y; Nagano,N;Okita,Y;Izumido,H;Sugimoto,S;Hayoshi,M. (2011).Effect of Tween 80 on the growth, lipid accumulation and fatty acid composition of*Thraustochytrium aureum*ATCC 34304.*Jornal of bioscience and bioengineering.*Vol111No.4, 42-424.

Ulrich M. (2008). The Global Market for Carotenoids, *BBC Market Research*

Valduga E, Tatsch P, Vanzo L, Rauber F, Di Luccio M, Treichel H.(2009). Assessment of hydrolysis of cheese whey and use of hydrolysate for bioproduction of carotenoids by *Sporidiobolus salmonicolor* CBS 2636.*J Sci Food Agric*,89:1060–1065

Wang SL, Zhang X, Zhang H, Lin K. (2001). Effects of some additives on the growth and carotenoids content of Rhodotorula. *Food Sci Technol* 2:20–2

Wang, S. L; Sun, J. S; Han, B. Z; Wu, X. Z. (2007). Optimization of β-Carotene Production by *Rhodotorula glutinis* Using High Hydrostatic Pressure and Response Surface Methodology. *Journal of food science an official ication of the Institute of Food Technologists* 72(8): M325-M329

Wang ,S. Sun,J.Han,Z Wu,X. (2008). Enhanced β-carotene production by *Rhodotorula glutinis* using high hydrostatic pressure. *Korean J. Chem. Eng*.,25(3), 513-516

Wang,Q.,GUO,F.,Rong,Y.,Ming-chi, Z. (2012). Lipid production from hydrolysate of cassava starch by *Rhodosporidium toruloides*. *Renewable Energy* 46: 164-168.

Wu, S.G., Hu, C.M., Jin, J.G., Zhao, X., Zhao, Z.K. (2010). Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresour. Technol*. 101, 6124–6129.

Xu, J., Oura,T., Liu, D.,Kajiwara,S. (2011). Heat-alkaline treatment of excess sludge and the potential use of hydrolysate as nitrogen source for microbial lipid production, Chin. *J. Biotechnol*. 27:482–488.

Yamane Y, Higashida K, Nakashimada Y, Kakizono T, Nishio N.(1997). Astaxanthin production by *Phaffia rhodozyma* enhanced in fed-batch culture with glucose and ethanol feeding. *BiotechnolLett* 19:1109–1111.

Ye Z. (2008). Biosynthesis and regulation of carotenoids in *Dunaliella* : progressesand prospects. Biotechnol Adv 26:352–360

Ykema, A., R.H.A.Bakels, I.I.G.. Verwoert, H. Smit and H.W.V.Verseveld. (1989). Growth yield, maintenance requirements, and lipid formation in the oleaginous yeast *Apiotrichum curvatum*. *Biotechnol. Bioeng*. 34:1268-1276.

Yoon SH. (2009). Combinatorial expression of bacterial whole mevalonate pathway for the production of beta-carotene in E. coli . *J Biotechnol* 140:218–226.

Zhao Z, Li Y ,Bai F. (2007). High-density cultivation of oleaginous yeast *Rhodosporidium toruloides* Y4 in fed-bach culture. *Enzyme Microb Te*chnol 41:312–317.

Zhao X, Wu S, Hu C, Wang Q, Hua Y, Zhao ZK. (2010). Lipid production from Jerusalem artichoke by *Rhodosporidium toruloides* Y4. *J Ind Microbiol Biotechn*ol 37:581–585.

Zhang, D.H., and Lee, Y.K.(2001).Two-Step Process for Ketocarotenoid Production by a Green Alga, Chlorococcumsp. *Strain MA-1, Appl. Micro. Biotech*. 55: 537–540

CURRICULUM VITAE

Name Surname: Furat Alakraa

Place and Date of Birth: HOMS- SYRIA 01.09.1985

Address: Menderes mah.363 No.16 D.6 Esenler-ISTANBUL

E-Mail: furat-alakraa@hotmail.com

B.Sc.: Albaath University- Food Engineering Department

Professional Experience and Rewards: Practical instructor at Science faculty in Albaath University

July 2009- September 2010